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The *S. Cerevisiae* Rho GTPase Cdc42p Is Spatially Regulated by Complex Formation with its GDI Rdi1p and Localization of its GEF Cdc24p

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THE *S. cerevisiae* RHO GTPase Cdc42p IS SPATIALLY REGULATED
BY COMPLEX FORMATION WITH ITS GDI Rdi1p
AND LOCALIZATION OF ITS GEF Cdc24p

A Dissertation Presented

by

Karen C. Cole

to

The Faculty of the Graduate College

of


The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Microbiology and Molecular Genetics


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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy specializing in Microbiology and Molecular Genetics.

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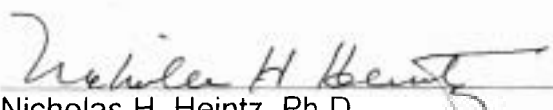
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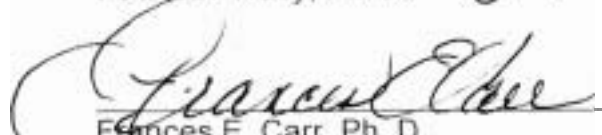
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Date: May 14, 2008

Abstract

The *S. cerevisiae* Rho GTPase Cdc42p localizes around the entire plasma membrane, but is only activated at sites of polarized growth in a cell cycle-dependent manner. This spatial and temporal control ensures that Cdc42p is inactive within the cytoplasm and outside of the polarized cortical domain, and is active at sites of polarized growth. This dissertation addresses spatial control of Cdc42p by examining complex formation with its sole guanine nucleotide dissociation inhibitor (GDI) Rdi1p and its sole guanine nucleotide exchange factor (GEF) Cdc24p. Rdi1p is not essential; however, when overexpressed, it is lethal, suggesting that it does have an important negative regulatory function. To study complex formation and localization, the technique of bimolecular fluorescence complementation (BiFC) was used to study *in vivo* Rdi1p-Cdc42p complex formation and localization, and how mutations to Rdi1p's two functional domains, the regulatory arm and the geranylgeranyl binding pocket, affected the complex. Rdi1p and Cdc42p interacted diffusely throughout the cytoplasm of cells at all stages of the cell cycle and their interaction was enhanced at incipient bud sites, the tips and sides of small- and large-budded cells and at the mother-bud neck region. Mutant Rdi1p was still able to interact with Cdc42p; however, the complex was absent from the cytoplasm. The Cdc42p Switch II domain mutation has the same phenotype as the Rdi1p mutations and, surprisingly, Cdc42p without a geranylgeranyl modification can still interact with Rdi1p in the cytoplasm, although with decreased affinity. These results are consistent with Rdi1p extracting Cdc42p from sites of polarized growth, which is dependent on both of its functional domains, and sequestering Cdc42p in the cytoplasm, where it is inactive. Cdc42p is also activated at incipient bud sites, tips of small buds and the mother-bud neck by the precise targeting of Cdc24p. This research also sought to identify protein(s) and regulatory mechanisms that function in targeting and maintenance of Cdc24p at polarized growth sites. The GFP-tagged targeting and anchoring domain of Cdc24p (aa 647-854) was used to screen haploid deletion mutants for localization to polarized growth sites. *boi2Δ*, *ent2Δ*, *hua1Δ*, and *rsc2Δ* mutants did not target GFP-Cdc24-647-854p to sites of polarized growth; therefore, Boi2p, Ent2p, Hua1p and Rsc2p have direct or indirect roles in targeting and maintenance of Cdc24p to sites of polarized growth. *skg6Δ* *tos2Δ* mutants abnormally localized GFP-Cdc24-647-854p pre-anaphase to the mother-bud neck. The same abnormal localization of GFP-Cdc24-647-854p was seen when potential CDK phosphorylation sites were mutated. This indicates that Tos2p, and its homolog Skg6p, and phosphorylation of the Cdc24p targeting and anchoring domain, are important for the maintenance of Cdc24p at sites of polarized growth. This dissertation research contributes to our understanding of how Cdc42p regulators affect its localization and activity by studying the spatial and temporal localization of the Cdc42p-Rdi1p complex and by identifying protein(s) involved in targeting and maintenance of Cdc24p at sites of polarized growth.

Citations

Cole, K. C., H. W. McLaughlin, and D. I. Johnson. 2007. Use of bimolecular fluorescence complementation to study in vivo interactions between Cdc42p and Rdi1p of *Saccharomyces cerevisiae*. *Eukaryotic Cell* **6**:378-387.

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CHAPTER 1

Introduction

Nomenclature

Table 1: Standardized nomenclature.

Researchers in the *Saccharomyces cerevisiae* field use the following standardized nomenclature.

	Convention	Example
Genes	Three letter name followed by a number	Wild-type: <i>CDC42</i> Mutant: <i>cdc42</i>
Alleles	Number separated from the gene with a hyphen or amino acid change in ()	<i>cdc42-116</i> ; <i>cdc42(R66E)</i>
Temperature-sensitive Alleles	Number separated from the gene with a hyphen, ^{ts}	<i>cdc24-4^{ts}</i> , <i>cdc24-Ts</i>
Proteins	Identical to gene name, first letter capitalized and followed by 'p'	Wild-type: Cdc42p Mutant: Cdc42-R66Ep
Δ	Disruption or deletion of genes	<i>cdc42\Delta</i>
Uncharacterized ORF	Y(A...P)R/L XX w/c: Y yeast, (A...P) 16 chromosomes, R/L right/left chromosome arm, XX ORF start from centromere, w/c Watson or Crick strand	YHR149c

Table 2: Cellular functions associated with gene abbreviations.

This table lists the cellular functions of genes that will be frequently mentioned in this dissertation.

Gene Abbreviation	Function
<i>AXL</i>	Axial budding
<i>BEM</i>	Bud emergence
<i>BUD</i>	Bud-site selection
<i>CDC</i>	Cell division cycle
<i>EXO</i>	Exocyst
<i>SEC</i>	Secretion

Table 3: Commonly Used Abbreviations.

Commonly used abbreviations within this dissertation and their designations.

Abbreviation	Designation
BiFC	B imolecular F luorescence C omplementation
CH	C alponin H omology
CRIB	C dc42/ R ac I nteractive B inding
CDK	C yclin- D ependent K inase
DH	D bl H omology
ENTH	E psin N -terminal H omology Domain
GFP	G reen F luorescent P rotein
GAP	G TPase A ctivating P rotein
GDP	G uanine nucleotide D i P hosphate
GDI	G uanine nucleotide D issociation I nhibitor
GEF	G uanine nucleotide E xchange F actor
GTPase	G uanine nucleotide T ri P hosphatase
GTP	G uanine nucleotide T ri P hosphate
MAPK	M itogen A ctivated P rotein K inase
PAK	p 21-activated K inase
PE	P hosphatidyl e thanolamine
PI	P hosphatidylinositol
PI(4,5)P ₂	P hosphatidylinositol 4,5-bis p hosphate
PS	P hosphatidyl s erine
PH	P leckstrin H omology
Rho	R as H omology
SGD	S accharomyces G enome D atabase

***S. cerevisiae* Polarity, Morphogenesis and Cell Cycle**

Cell polarity arises because of the asymmetric distribution and maintenance of cellular components, which results in the formation of structurally and functionally distinct membrane domains. These membrane domains are essential for cell motility (240), embryogenesis (317), stem cell (154) and neuronal (183) differentiation, organogenesis (148, 149), immune synapse formation (39, 265), phagocytosis (32), leukocyte chemotaxis (32, 265), and T cell differentiation (261). Although different cell types and morphologies are involved, the molecular pathways that organize them are conserved from yeast to man (211). Polarity establishment begins with an intrinsic or extrinsic positional cue that is recognized and amplified through a positive feedback loop, resulting in reorganization of the actin cytoskeleton, spindle orientation and asymmetric protein localization.

S. cerevisiae grows asymmetrically, establishing a form of apical-basal cell polarity that directs cell growth into the bud (intrinsic cue) and to orient towards a mating partner after pheromone stimulation (extrinsic cue). This dissertation focuses on the cell cycle, polarity and morphogenesis events related to budded growth of haploids (unless otherwise noted). Polarization of the actin cytoskeleton into the bud is coordinated with the cell cycle (**Figure 1**) to ensure the fidelity of mitotic spindle orientation and organelle inheritance and to target secretory vesicles to discrete sites on the plasma membrane for delivery of cell wall remodeling enzymes and new membrane to facilitate apical bud growth [reviewed in (237)].

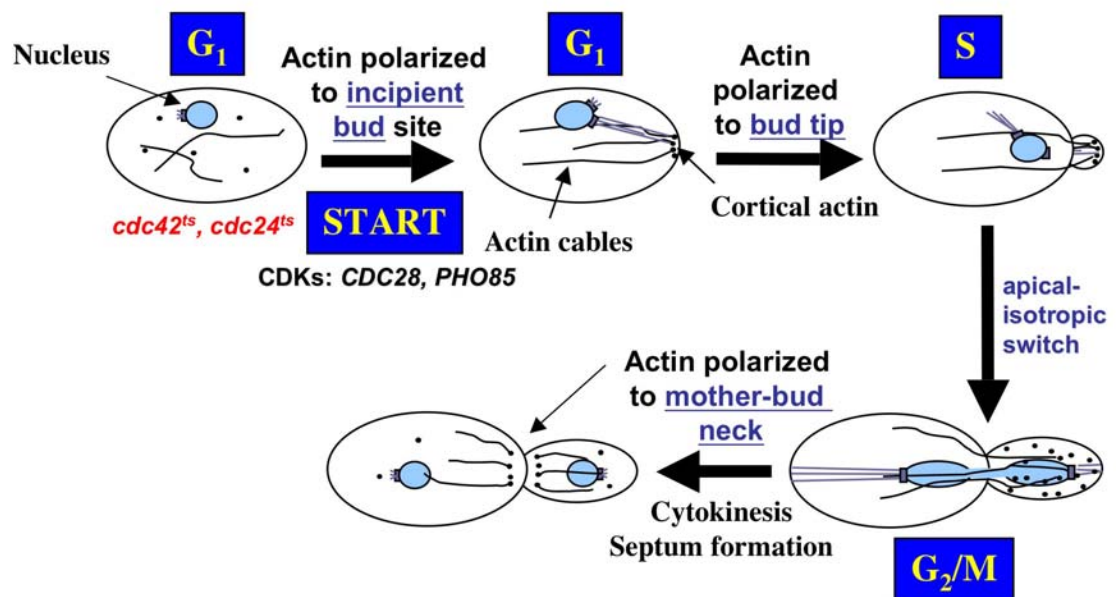
Morphogenesis and the Actin Cytoskeleton

S. cerevisiae is surrounded by a rigid cell wall consisting of glucan, mannan and chitin that provides shape and environmental protection [reviewed in (167)]. The shape of budded yeast (**Figure 1**) is controlled by a combination of cell wall deposition, which occurs in cycles of spatially and temporally controlled isotropic and polar growth, and turgor pressure (115). *S. cerevisiae* undergo isotropic growth in G₁ until a critical size is reached. Because they grow asymmetrically, this ensures that daughter cells attain the same size as mother cells (260). Cells can then commit to cell cycle entry (pass START) and select a bud site to which the actin cytoskeleton will become polarized (**Figure 1**). Patches, cables and rings, the three filamentous actin structures in *S. cerevisiae*, are organized and localized in a cell cycle-dependent manner [reviewed in (204)] that is also dependent on the Rho GTPase Cdc42p (145). In *cdc42^{ts}* mutants, cortical actin patches and actin cables still form; however, they are randomly oriented, resulting in isotropic growth and large, round, unbudded cells due to an inability to polarize growth (2).

Cortical actin patches are 150-250 nm membrane invaginations that co-localize with actin and actin-binding proteins (205) [see **Diffusion Restriction**]. Actin patches are nucleated at the cell cortex by the Arp2/3 complex (204). The bud emerges through a ring of cortical actin patches and then cortical patches form in the bud, where they exhibit a predominately random localization, with some

Figure 1: Morphological changes occur through cell-cycle dependent reorganization of the actin cytoskeleton.

S. cerevisiae in G₁ grow isotropically until a critical size is reached. At this time, cells pass START and establish polarity by aligning actin cables along the mother-bud axis. Cortical actin patches form a ring at the incipient bud site, through which the bud emerges, and are maintained at the tip of the emerging bud. After the apical/ isotropic switch, the actin cables and patches directing cell growth must be retargeted to the mother-bud neck for cytokinesis and septum formation. *cdc42^{ts}* and *cdc24^{ts}* cells are unable to establish apical growth and arrest in G₁ as large, round unbudded cells.



clustering near sites of polarized growth (9).

Actin cables are nucleated independently of the Arp2/3 complex at the cell cortex by two formins [reviewed in (80)]: Bni1p at the bud tip and at the mother-bud neck by Bnr1p (204). The formin FH2 domain binds the barbed end of the growing actin filament; therefore, actin cables elongate from the cortex to the cell interior (37). Formation of cables at these sites ensures that secretory vesicles are directed to sites of polarized growth (237, 238) [see **Targeted Vesicle Delivery**]. Bni1p then moves from the bud tip to the mother-bud neck at anaphase (223) to facilitate Rho1p-dependent assembly of actin in the actomyosin ring (295) [see **Redirecting Polarized Growth to the Mother-Bud Neck**].

Cell Cycle Regulation of Establishment and Maintenance of Cell Polarity

The five cyclin-dependent kinases (CDKs) of *S. cerevisiae* (Cdc28p, Pho85p, Kin28p, Ssn3p and Ctk1p) are proline-directed kinases (ProDKin) that phosphorylate Ser and Thr residues in target proteins to regulate progression through the cell cycle (192). Kin28p, Ssn3p and Ctk1p phosphorylate the carboxyl-terminal domain (CTD) of RNA polymerase II, resulting in transcription initiation. Cdc28p (also referred to in the literature as Cdk1p) is principally responsible for cell cycle transit. Pho85p, which is 51% identical to Cdc28p, has a secondary role in the cell cycle (200).

Cdc28p (consensus phosphorylation sequence: S/T-P-X-K/R) interaction with the G₁ cyclins (Cln1-3p) is required for progression to S phase (START) [reviewed in (26)]. Since expression of only one of the three G₁ cyclins is essential, cells can be arrested in G₁ by cyclin depletion and released synchronously by expression of one cyclin (see **Chapter 2**). In addition to phosphorylation events directed by Cdc28p – cyclin interactions, the transcription factors SBF and MBF initiate mRNA production of many genes in late G₁, which are essential for cell cycle progression (*CLN1* and *CLN2* by SBF, *CLB5* and *CLB6* by MBF) and maintenance of cell polarity (*TOS2* by SBF, see **Chapter 3**).

Cdc28p-dependent phosphorylation is additionally required for maintenance of bud growth (190). Inhibition of Cdc28p activity results in a loss of polarized localization of Bem1p, Boi1p, Boi2p and Rga2p, with a concomitant decrease in the phosphorylation of Boi1p, Boi2p, Rga2p and Cdc24p (190) (see **Chapter 3**). Since all of these proteins either interact with, or regulate Cdc42p, Cdc28p-dependent phosphorylation is an essential component of connecting the cell cycle with polarized growth.

Progression through S phase requires Cdc28p interaction with the cyclins Clb5p and Clb6p. The mitotic cyclins Clb1-4p can substitute for Clb5-6p, although a delay in initiation of DNA replication occurs [reviewed in (26)]. Far1p is the cyclin-dependent kinase inhibitor (CKI) of cyclin-Cdc28p complexes that is required for pheromone-induced cell cycle arrest in mating cells [reviewed in (192)]. Additionally, Far1p interacts with the CH domain of Cdc24p [see **GEF**

Functional Domains] to sequester Cdc24p in the nucleus. Cdc28p phosphorylation of Far1p leads to its degradation (276) and nuclear export of Cdc24p to sites of polarized growth (212, 276). ***The protein(s) responsible for the specific targeting of Cdc24p to sites of polarized growth are a focus of this dissertation research and are discussed in Chapter 3.***

Pho85p (consensus phosphorylation sequence: S/T-P-X-I/L) regulates stress adaption and cell wall integrity signaling (131) while interacting with the Pho80 cyclins (Pho80p, Pcl6-8p and Pcl10p), and exerts its cell cycle activity while interacting with the Pcl 1,2 cyclins (Pcl1-2p, Clg1p, Pcl5p and Pcl9p) (200). A total of 53 genes are synthetic lethal (SL) in a *pho85Δ* mutant; of these *BNI1*, *CLN1CLN2*, *CLA4*, *BEM1*, *BEM2*, and *BEM4* have direct roles in the establishment and maintenance of cell polarity (131). A total of 769 genes are synthetic dosage lethal (SDL - lethal when overexpressed) in a *pho85Δ* mutant, of these *BMH1*, *BNI1*, *BNI4*, *AXL2*, *CDC3*, *GIC1*, *GIC2*, *RDI1*, *RGA2*, and *STE20* have direct roles in the establishment and maintenance of cell polarity (283). An SL or SDL phenotype indicates that these proteins may be targets of Pho85p, and that phosphorylation by Pho85p plays a role in the regulation of their polarity functions.

LG1C- cells (late-G₁ cyclinΔ mutant *cln1Δ cln2Δ pcl1Δ pcl2Δ* that is rescued from G₁ arrest by expression of *PCL2* from a galactose-inducible promoter) have severe morphological defects that can be attributed to bud emergence, new septin ring assembly and old septin ring disassembly defects

(199). The bud emergence defect arises because these cells are unable to target Cdc24p to the incipient bud site. Taken together, these observations indicate that Cdc28p- and Pho85p-dependent phosphorylation of cell polarity determinants affects their activity, localization, and the establishment and maintenance of cell polarity and growth (see **Chapter 3**).

Rho GTPases

Rho GTPases are 21 kD monomeric G-proteins that are the primary effectors of actin cytoskeleton rearrangements. Rho GTPases are spatially and temporally controlled through interaction with three classes of regulatory proteins (**Figure 2**): Guanine nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and Guanine nucleotide Dissociation Inhibitors (GDIs) (30).

This dissertation addresses spatial control of the *S. cerevisiae* Rho GTPase Cdc42p by examining complex formation with its GDI (Rdi1p) (see Chapter 2) and localization of its GEF (Cdc24p) (see Chapter 3).

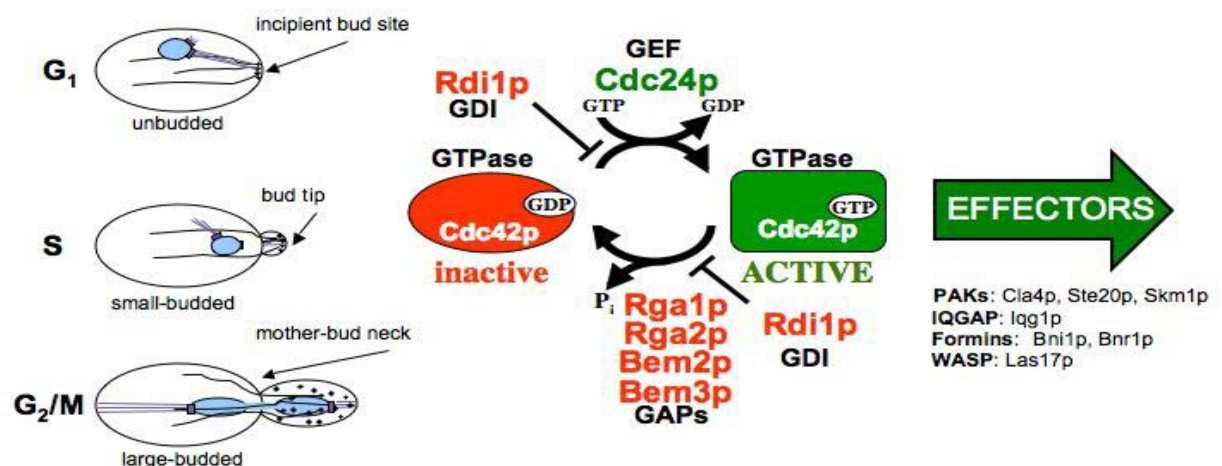
GTPases function as binary molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. When active, Rho GTPases activate signaling pathways that regulate polarized cell growth and morphogenesis (145), cell cycle progression (143, 313), gene transcription (143), vesicle trafficking (77, 251, 252), cell migration (240), enzyme activation

Figure 2: Regulatory proteins control the activity of the Rho GTPase Cdc42p.

Regulatory proteins are targeted, maintained, and removed from sites of polarized growth (indicated by **arrows**) in a cell-cycle dependent manner. Cdc42p is localized around the entire plasma membrane throughout the cell cycle. Cdc42p is activated specifically at sites of polarized growth by the targeting of its GEF Cdc24p and inactivated by targeting of its GDI Rdi1p and its GAPs. Targeting of positive and negative regulators effects Cdc42p interaction with, and subsequent activation of, its downstream effector proteins.

Regulators that result in an active GTPase are **green** (GEFs activate GTPases by catalyzing the exchange of GDP for GTP) and regulators that result in an inactive GTPase are **red** (GAPs and GDIs inactivate GTPases by stimulating intrinsic GTPase activity and extracting from cellular membranes, respectively).

The morphology of cells at all stages of the cell cycle is shown along with their phenotypic characterization as unbudded, small-budded or large-budded cells. The G₁ arrow points to the **incipient bud site**, the S arrow to the **bud tip** and the G₂/M arrow to the **mother-bud neck**, designations that are used throughout this dissertation.



(71, 134), apoptosis (31), and ion channel regulation (236). Given their important roles, Rho GTPases, their regulators and effectors are often the targets of bacterial virulence factors [reviewed in (7, 103)] and they are found in the α -synuclein aggregates associated with Parkinson's disease, which likely prevents their functioning in signaling pathways, resulting in neurodegeneration (65). Aberrant Rho GTPase signaling via overexpression of the GTPase and its regulatory and effector proteins or activation via deregulated growth factors is often seen in human cancers (262); however, the only *rho* mutation found in human cancer is RhoH in some patients with non-Hodgkin's lymphoma [reviewed in (29)]. Defects in the normal functioning of a RhoGAP, RhoGEF and the Rho GTPase effector PAK3 are found in X-chromosome linked forms of mental retardation (XMR), a RhoGEF is implicated in Down syndrome, faciogenital dysplasia (FGD, Aarskog-Scott syndrome) and amyotrophic lateral sclerosis (ALS), and the Rho GTPase effectors Wiskott-Aldrich syndrome protein (WASP) and Diaphanous cause immune system dysfunction and non-syndromic deafness, respectively (reviewed in (29)).

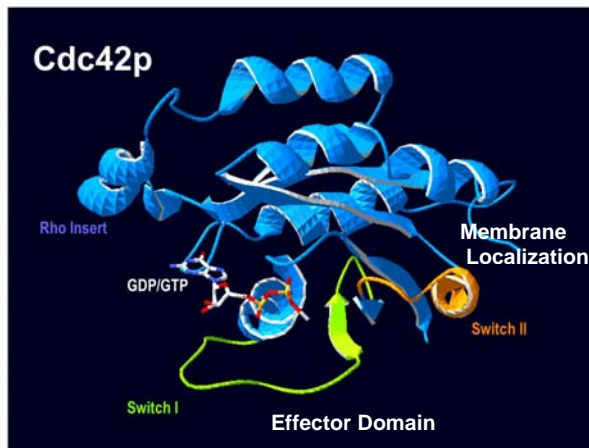
GTPase Structure and Interaction with Regulators and Effectors

The G-domain fold, consisting of a six-stranded β -sheet and α -helices that are characteristic of these proteins, is illustrated in the homology-modeled

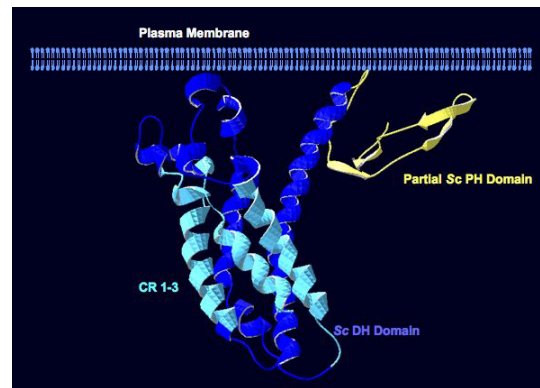
Figure 3: Homology-modeled structure of Cdc42p and Cdc24p.

A. SWISS-MODEL-generated structure of *S. cerevisiae* GDP-bound Cdc42p [(61), **Chapter 2**] with the Effector Domains Switch I (**lime**) and Switch II (**orange**) and carboxyl-terminal membrane localization domains highlighted. The Rho insert domain, which distinguishes RHO from other members of the Ras GTPase family, is also identified. **B.** The magnification below illustrates Cdc42p amino acid residues that are important for GDP/GTP exchange in conserved regions G1 –G4. **C.** A SWISS-MODEL-generated structure of the *S. cerevisiae* DH (**blue**) and partial PH domains (**yellow**) is shown correctly oriented with the plasma membrane. CR 1-3 (**light blue**), which stabilize the DH domain and interact with Cdc42p Switch II domain, are highlighted.

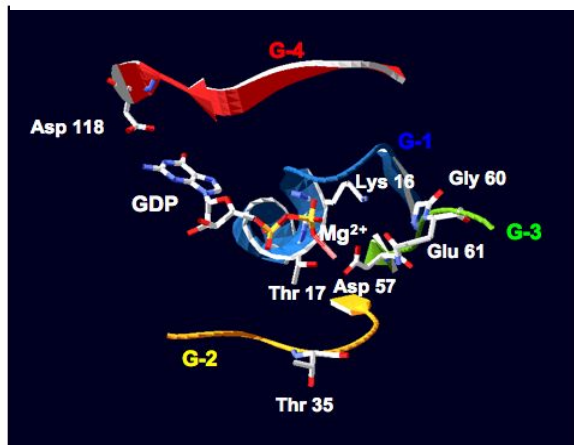
A. Structure of *S. cerevisiae* Cdc42p



C. Structure of *S. cerevisiae* Cdc24p catalytic DH domain and partial PH domain



B. Magnification of the nucleotide-binding pocket



structure of the *S. cerevisiae* Rho GTPase Cdc42p (305) (**Figure 3**). Regions critical for GDP/GTP exchange are highlighted in the magnification in **Figure 3** (G-1 – G-4) [reviewed in (35)]. In G-1, Lys16 interacts with the α - and β -phosphates of GTP or GDP while Thr17 interacts with the β - and γ -phosphates of GTP. Additional amino acids also play a role in nucleotide coordination: the G-3 Asp57 coordinates Mg^{2+} through a water molecule and Gly60 forms a hydrogen bond with the γ -phosphate of GTP, and in G-4, Asp118 forms a hydrogen bond with the guanine ring.

The exchange of GDP for GTP results in the conformational change of Switch I (G-2, see following and **Figure 3B**) and Switch II (see **Figure 3A**) in most GTPases (62, 67, 213, 226). These regions interact with GEFs, GAPs, GDIs, and downstream effector proteins, which are ultimately responsible for the cellular effects outlined above. Switch regions are termed ‘multispecific’ because the binding sites for regulatory and effector proteins overlap (24). *in vitro* studies of Cdc42 indicate that in addition to GDP – GTP exchange, binding of effector proteins is required to induce conformational change in Switch I (233).

When GDP is bound, Thr35 in G-2 (crystal structure shows GDP-binding state in **Figure 3B**) flips away from the nucleotide; however, when GTP is in the nucleotide-binding pocket, Thr35 flips inwards and interacts with both the Mg^{2+} ion that is required for GTP hydrolysis and the γ -phosphate of GTP. The orientation change of Thr35 is likely to induce other changes within the Switch I

domain (35) to facilitate (GTP-bound) or prevent (GDP-bound) interaction with effector proteins.

Dbl-family GEFs (Cdc24p in *S. cerevisiae*) have three conserved helices in their DH domains (CR 1-3) which are highlighted in the partial homology modeled structure of Cdc24p in **Figure 3C**. Rho GTPase Switch I interacts with CR1 and CR3 and Switch II interacts with CR3 (257). This GEF – GTPase complex results in changes to the nucleotide-binding pocket that facilitate release of the bound GDP and reloading with GTP. The RhoGAP domain interacts with Cdc42p Switch I and II domains and inserts an arginine residue (arginine finger) into the nucleotide-binding pocket to interact with Glu61 (see **Figure 3B**). The arginine finger catalyzes the intrinsic GTPase ability of Cdc42p, resulting in the hydrolysis of the active GTP-bound Cdc42p to the inactive GDP-bound Cdc42p (111). The amino-terminal regulatory arm of GDIs (see **GDIs**) interacts with the Switch I and II domains of Rho GTPases to prevent GTP hydrolysis, GDP exchange and interaction with effector proteins (124).

Rho GTPase effector proteins often contain a Cdc42- and Rac-interactive-binding (CRIB) binding domain [also referred to in the literature as PBD (p21 binding domain)]. When GTP-bound GTPase Switch I (effector domain) interacts with CRIB domains, the result is a structural change in the effector protein that relieves its autoinhibited state (123).

The *S. cerevisiae* Rho GTPase Cdc42p

Mammals have 23 Rho GTPases, 85 Rho GEFs, 70 Rho GAPs (112) and 3 Rho GDIs (72). There is little specificity of one regulator for one GTPase, which makes discerning the function of any one regulator on any one Rho GTPase virtually impossible. However, in *S. cerevisiae*, Cdc42p has only one GEF (Cdc24p), one GDI (Rdi1p) and four GAPs (Rga1-2p and Bem2-3p). Cdc42p shares 80-95% amino acid identity with Cdc42 in other organisms (145). Since these proteins are highly conserved, studying regulators of Cdc42p and their direct effects on cell polarity in yeast is much more productive, and can often be applied directly to other organisms.

S. cerevisiae has six Rho GTPases: Rho1-5p and Cdc42p (there is no Rac homolog). Only Rho1p and Cdc42p are essential for viability – Rho1p is the central regulator of cell wall integrity signaling (173) and Cdc42p the principal regulator of the actin cytoskeleton and cell polarity (145, 228). **Table 4** lists the *cdc42* alleles that have been identified to illustrate the role of Cdc42p in functions outside of its role in the establishment and maintenance of polarization, which is the focus of this dissertation. Therefore, other roles of Cdc42p will not be discussed.

Table 4: *S. cerevisiae* cdc42 alleles illustrate varied functions of Cdc42p.

Altered Function	cdc42 Alleles	Phenotype(s)	Disrupted Interaction(s)	Reference(s)
Nucleotide Exchange	G12V, T17N, T35A, Q61L, S86P, I117S, D118A, T138A, L165S, K186R, -116, -117, -142, -125	Large round unbudded, hyperpolarized, lethal, elliptical	Cdc24p, GAPs, Rdi1p	(61, 66, 160, 194, 332, 333)
Membrane Localization	C188S, K183-187Q	Large, round, unbudded	regulators and effectors	(66, 247, 332, 333)
Morphogenesis Checkpoint	V44A, V36T	Hyperpolarized multibudded, multinuclear	Cla4p, Gic1p, Gic2p	(160, 248)
Budding Frequency	G60A	multibudded	Cdc24p	(48)
Polarity Establishment	-101, -118	Large round unbudded	Cdc24p	(160)
Polarity Maintenance	D38E	Hyperpolarized multibudded	Cla4p, Bem3p	(246)
Exocytosis	-6, -123, -13, -201	Vesicle accumulation in small-budded cells, unbudded or multibudded with no exocyst targeting	Sec3p	(1, 206, 327)

Altered Function	<i>cdc42</i> Alleles	Phenotype(s)	Disrupted Interaction(s)	Reference(s)
Haploid Invasive Growth	I46M, S71P	Non-invasive	effector proteins	(203)
Diploid Pseudohyphal Growth	N26I, R68S, E100G, S158T, L160P, A30T, D65N, N92D, E95K	Non- or Hyper-filamentous	effector proteins	(203)
Loss of function	Y32K, F37A, Y40C	Lethal	Igq1p, Ste20, Cla4p, Gic1p, Gic2p	(246)

Cdc42p Polarity Effector Proteins

Cdc42p interacts with the following CRIB domain proteins: the PAKs Cla4p, Ste20p and Skm1p and the GTPase-interacting component proteins Gic1p and Gic2p. Cla4p has multiple functions when activated by Cdc42p. Several of these functions which will be mentioned later in this dissertation are: formation of the septin collar at the mother-bud neck (304), participation in the G₂/M transition by phosphorylation of Swe1p (Wee1 homolog) (168), role in mitotic exit (55) and phosphorylation of Cdc24p (106). *cla4Δ* mutants are viable, but have a cytokinesis defect (64). Ste20p primarily activates pheromone response and filamentation signaling pathways (166); however, it does have some role in bud emergence and growth (127). *cla4Δ ste20Δ* mutants are unable to maintain septin rings at the mother-bud neck and do not undergo cytokinesis (64). *SKM1* is not redundant with either *CLA4* or *STE20* and *skm1Δ* strains have no evident phenotype; however, overexpression results in morphological defects (187), indicating some role in polarized growth and morphogenesis.

Gic1p and Gic2p, which have no significant homology to other proteins (53), are essential for polarization of the actin cytoskeleton (40), have a role in septin ring formation at the incipient bud site (141) and function in mitotic exit in the absence of *CDC5* and *LTE1* (126). *gic1Δ gic2Δ* mutants have a slow growth phenotype at 30°C (40), suggesting that other Cdc42p effector proteins can establish polarized growth in their absence. It has been proposed that Gic1p and Gic2p are redundant with a Bni1p-dependent pathway (23).

Bni1p and Bnr1p are the two yeast formins responsible for nucleation of actin cables at the cell cortex. Binding of mammalian RhoGTPases to the formin GT Pase-binding domain (GBD) relieves an inhibitory intramolecular interaction between it and the Dia autoregulatory domain (DAD) [reviewed in (80)], analogous to the interaction between GTPases and CRIB domains [see **GTPase Structure and Interaction with Regulators and Effectors**]. Although this mechanism of formin regulation has not been demonstrated in yeast (204), Bni1p does interact with Cdc42p via its Rho-insert domain (249) and formin-mediated actin cable assembly is regulated at different times in the cell cycle by Cdc42p, Rho1p, Rho3p and Rho4p (204).

The Cdc42p essential effector protein Iqg1p (IQGAP homolog) is required for axial budding and cytokinesis and has a role in targeted secretion to sites of polarized growth by interacting with another Cdc42p effector, Sec3p (221) [see **Targeted Vesicle Delivery**]. The polarisome complex consists of Spa2p, Bni1p, Bud6p and Pea2p (275). Spa2p localization to sites of polarized growth is dependent on COPI-mediated vesicle transport of Cdc42p, but independent of Cdc24p (250), suggesting that localization of this effector does not initially require GTP-bound Cdc42p. Polarisome mutants form spherical buds and wide mother-bud necks, indicating that the function of this complex is to focus polarized growth to a discrete cortical site [reviewed in (237)].

Cdc42p localizes around the entire plasma membrane (247), but is only activated at sites of polarized growth. Therefore, to ensure that Cdc42p is active

(GTP-bound) only at sites of polarized growth to interact with downstream effector proteins, and inactive (GDP-bound) at all other sites, the proteins that regulate its activity must be specifically targeted to these sites. Their function and regulation are discussed below (see **Figure 2**).

Cdc42p Regulatory Proteins

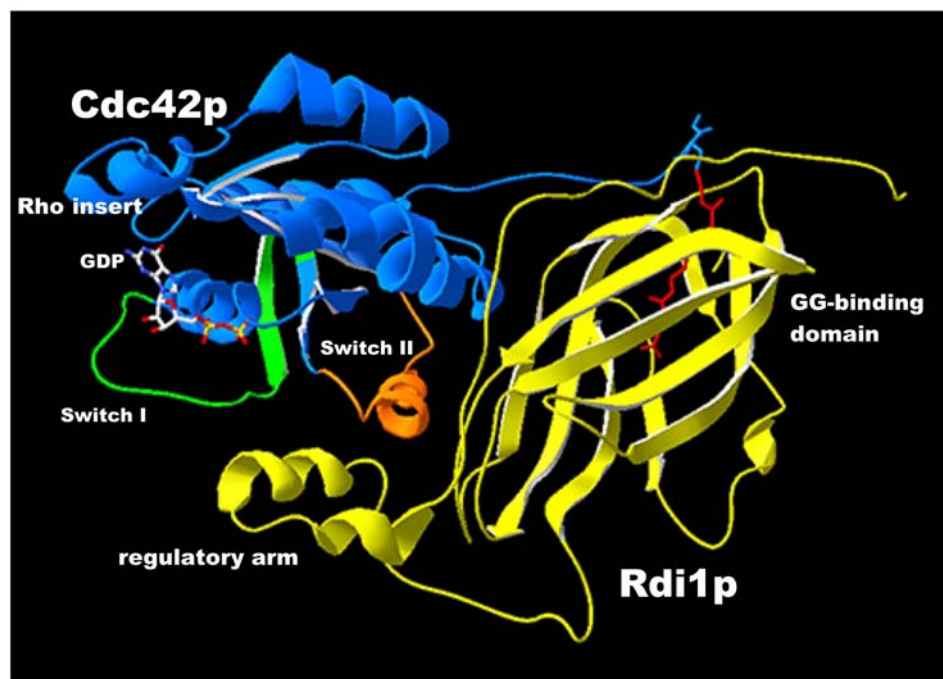
The Guanine-Nucleotide Dissociation Inhibitor (GDI) Rdi1p

RhoGDIs consist of two functional domains: an amino-terminal regulatory domain that remains largely unstructured unless the GDI is complexed with a Rho GTPase (101) as shown in **Figure 4**, and a carboxyl-terminal geranylgeranyl (GG)-binding domain, which binds the prenyl moiety bound to the carboxyl terminus of the GTPase (124) resulting in its extraction from membranes (124, 235). GDIs are therefore mostly folded (large GG-binding domain) with local disorder (regulatory arm). This topology likely allows GDIs to recognize their GTPase targets with high specificity and low affinity (76), permitting them to dissociate after the GTPase is released to the membrane.

Current models (72, 219) for the GDI-dependent extraction of Rho GTPases from membranes are based on studies in mammalian cells. The cytosolic GDP-bound GTPase–GDI complex is translocated to the plasma membrane where a displacement factor facilitates the release of the GTPase to the plasma membrane. GEFs then activate the GTPase at the membrane to

Figure 4: Homology-modeled structure of the Cdc42p – Rdi1p complex.

The SWISS-MODEL first-approach mode was utilized to build a homology model of the *S. cerevisiae* Cdc42p-Rdi1p complex from the template structure (PDB accession number 1DOA) of the human GDP-bound Cdc42-bovine RhoGDI complex. The Switch I (lime) and II (orange) domains of Cdc42p (blue) interact with the amino-terminal regulatory arm of Rdi1p (yellow) while the carboxyl-terminal geranylgeranyl (GG) moiety (red) is inserted into the GG-binding domain. See **Chapter 2**.



permit signaling to downstream effector proteins. GDIs, which bind GTP- and GDP-bound GTPases with equal affinity (214) can then extract either from the membrane to abrogate signaling or to recycle back to the membrane, respectively.

Rdi1p, the only GDI in *S. cerevisiae*, is not essential for growth, mating or budding pattern; however, it is lethal when overexpressed (188, 292) because Cdc42p (155, 249, 292) and Rho1p (155, 292), the two essential Rho GTPases, are inappropriately removed from the plasma membrane. Cdc42p (155, 292), Rho1p and Rho4p (292) were co-immunoprecipitated with Rdi1p. The other members of the yeast Rho family, Rho2p, Rho3p and Rho5p, may not interact with Rdi1p because they were not co-immunoprecipitated with Rdi1p or extracted from the membrane upon Rdi1p overexpression (292).

Green fluorescent protein (GFP)-tagged Rdi1p is a generally cytoplasmic protein that localizes to the plasma membrane at the tips of small buds and the mother-bud neck (249). Cdc42p co-localizes with Rdi1p in the cytoplasm, the tips of small buds and the mother-bud neck, consistent with the model that Rdi1p extracts Cdc42p from sites of polarized growth and sequesters it in the cytoplasm. Rdi1p can extract GTP-bound Cdc42-G12Vp from membranes, but not the GDP-bound or apo-nucleotide Cdc42-D118Ap (292). The absence of Rdi1p-dependent extraction of Cdc42-D118Ap is not surprising given the fact that the two proteins do not interact (61) [see **Chapter 2**].

Phosphorylation of mammalian RhoA and Cdc42 results in greater interactions with RhoGDI and enhanced membrane extraction (83) and phosphorylation of GDI results in a decreased interaction with RhoA and Cdc42 (69). Little is currently known about the role phosphorylation of Rdi1p or Cdc42p has in determining their interaction. Rdi1p was identified in an *in vitro* proteome chip assay as a phosphorylation target of Yck1p (casein kinase I) and YPL141c (similar to Kin4p, a Ser/Thr kinase that inhibits the mitotic exit network (MEN)) (239), but these kinases have not been shown to have an effect on Rdi1p *in vivo*.

Overexpression of the PAK Cla4p blocks the interaction between Rdi1p and Cdc42p and results in a Rdi1p-dependent decrease in cytosolic Rho1p and Cdc42p (292). Cla4p is potentially playing an indirect role (*i.e.*, phosphorylation of another protein(s) that actually block the interaction between the GTPase and GDI) because direct phosphorylation of either the GTPase or GDI by Cla4p was not seen *in vitro* (292). Rdi1p, like mammalian GDIs, is likely to be regulated by direct or indirect phosphorylation to ensure that Cdc42p is down-regulated when polarized growth is no longer required (**see Conclusions and Future Directions**), although we currently know little about it.

The Guanine-Nucleotide Exchange Factor (GEF) Cdc24p

S. cerevisiae has four members of the Dbl (diffuse B-cell lymphoma) family of GEFs: Rom1p, Rom2p and Tus1p, the GEFs for Rho1-5p, and Cdc24p,

the only GEF for Cdc42p. Cdc24p is localized in a cell cycle-dependent manner. Cdc24p is sequestered in the nucleus of cells in G₁ by the CDKi, Far1p (212, 276). Cdc28p phosphorylates Far1p, resulting in its ubiquitin-mediated degradation (117) and Cdc24p release from the nucleus. Cdc24p also localizes to polarized growth sites: the incipient bud, tips of small-budded cells and the mother-bud neck post-anaphase (293). Cdc28p-dependent phosphorylation is required for Cdc24p localization at incipient bud sites (106), although whether this is a direct phosphorylation of Cdc24p at one of its six potential CDK phosphorylation sites, or phosphorylation of another protein that targets Cdc24p to the incipient bud site is still unknown.

Cdc42p is localized around the entire plasma membrane throughout the cell cycle and has enhanced localization or 'clustering' at sites of polarized growth (247). It is assumed that clustered Cdc42p is GTP-bound because this occurs at polarized growth sites, to which the GEF Cdc24p and Cdc42p effector proteins are specifically targeted. ***The mechanism responsible for translocation of Cdc24p to sites of polarized growth to activate Cdc42p is currently unknown and is one focus of this dissertation*** (see Chapter 3).

Functional Domains

Figure 5 is a linear representation of the functional domains present in Cdc24p and **Table 5** lists current Cdc24p alleles. Unfortunately, no complete crystal structure exists for any Rho GEF. The Cdc24p DH and PH domains are

Table 5: *cdc24* alleles

In contrast to the extensive information available on *cdc42* alleles (**Table 4**), few *cdc24* alleles are available. *cdc42^{ts}* and *cdc24^{ts}* alleles generally result in the same phenotype (large, round, unbudded cells); therefore, they both function in bud emergence.

Altered Function	<i>cdc24</i> allele(s)	Phenotype(s)	Disrupted Interactions	Reference(s)
Bud-site selection	<i>G168D</i> (-4 ^{ts}), -3	Random budding	Rsr1p	(277, 280)
Mating	<i>S189P</i> (-m3)	Mating defect	Far1p	(212)
	<i>R416G</i> (-m6)	Cell fusion defect	Fus1p, Spa2p	(18)
Non-functional	<i>W659A</i> , [<i>E653A</i> , <i>E654A</i> , <i>R656A</i>], -5	Large, round, unbudded	Cdc42p	(42, 294)
Bud tip maintenance	[<i>D824K</i> , <i>D831R</i>]		Bem1p	(138)
Unknown	<i>P150L</i> (-1 ^{ts})		unknown	(198)
Bud-site establishment	<i>G615S</i> , [1371V, <i>G423K</i>]	Large, round unbudded in high Ca ²⁺	unknown	(198)

48% identical to the DH and PH domains of the mammalian GEF ASEF [PDB 2PZ1] (197), which was used in the First Approach Mode of SWISS-MODEL (105, 270) to produce the homology-modeled structure of the complete Cdc24p DH domain and the partial PH domain (**Figure 3C**). Plasma membrane orientation of the model was determined by comparison to the probable orientation of the Dbs DH/PH–Cdc42 complex at the plasma membrane interacting with PI(4,5)P₂ (258).

Nuclear Export Sequence (NES)

Proteins that undergo nucleocytoplasmic shuttling have a nuclear localization sequence (NLS) and a nuclear export signal (NES). NLS motifs are usually a cluster of basic amino acids and one has not been identified in Cdc24p; therefore, it is likely to be imported via an interaction with another protein. Cdc24p does have a putative NES, aa 55-69, although mutation of this domain to confirm its function has not been done to date.

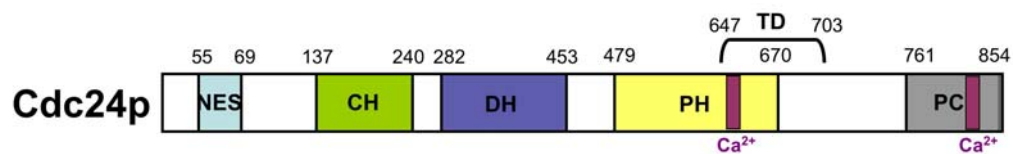
Calponin Homology (CH) Domain

Cdc24p is part of the CH3 family of proteins that have a single CH domain at their amino-terminus (157). SGD identifies only three additional yeast CH3 family members: Nop14p (nucleolar protein), Bim1p (binds microtubules), and Iqg1p (IQGAP). Although the CH domain was originally identified in the actin-binding protein calponin, there is no evidence that single CH domains bind actin

Figure 5: Cdc24p functional domains.

Linear representation of the functional domains present in Cdc24p.

NES Nuclear export sequence
CH Calponin homology domain
DH Dbl homology domain
PH Pleckstrin homology domain
TD Targeting domain
PC p40^{phox} and Cdc24p domain



Sequence of potential Ca²⁺ binding domain aa 649 – 658:

K F K N E E T R D N

Sequence of potential Ca²⁺ binding domain aa 820 – 831:

D E D G **D** F V V L G S **D**

Residues that would play a role in ion coordination are in **bold**. The two Asp residues that are important for Bem1p binding are indicated in **red**.

(94). *S. cerevisiae* does have two proteins with multiple CH domains, Sac6p and Scp1p, that function in actin bundling and cross-linking, respectively.

Deletion of the CH domain can't complement a *cdc24Δ* mutant, resulted in mislocalization from bud tips (in a *cln1Δ cln2Δ cln3Δ MET-CLN2* background), and interfered with Cdc24p function and interaction with Rsr1p (277). Taken together, this indicates that the CH domain of Cdc24p is likely to be a signaling module that may function similarly to other members of the CH3 family [see **Conclusions and Future Directions**].

Dbl Homology (DH) Domain and Pleckstrin Homology (PH) Domain

Cdc24p is a member of the Dbl-GEF family (256), which is characterized by tandem, carboxyl-terminal DH and PH domains (328). The DH and PH domains are considered to be the minimal functional unit to affect transformation of mammalian cells (330). There are approximately 70 human Dbl-GEFs, and each GEF can activate multiple Rho GTPases (88). Because Cdc24p is the only GEF for the Rho GTPase Cdc42p, it is easier to study the direct interactions of these proteins and their effects on cell polarity in *S. cerevisiae* [see **Figure 3C** for structure of DH domain and **GTPase Structure and Interaction with Regulators and Effectors** for a discussion of mechanism for DH-mediated GDP – GTP exchange].

Mammalian Dbl GEFs can be regulated by: autoinhibitory interaction between the DH and PH domain, or another signaling domain with the DH and

PH domains, DH domain oligomerization, and interactions with other proteins that regulate its function [reviewed in (328)]. In *S. cerevisiae*, an autoinhibitory, intramolecular interaction between the carboxyl-terminal PC domain (see **Functional Domains**) and another region within the targeting and anchoring domain has been proposed (277). *in vitro* chemical induction of DH domain oligomerization results in Cdc24p retention in the nucleus, but does not alter its GEF activity *in vitro* (195). This suggests that sequestering Cdc24p in the nucleus at different stages of the cell cycle (G_1 and post-anaphase) (293) could prevent activation of Cdc42p. The Rsr1p GTPase (294, 329), Bem1p (34, 294, 329) and Tos2p (86, 294), in addition to other proteins that interact with Cdc24p (see **Table 6** and **Table 7**) have a potential role in regulating the activity of Cdc42p by controlling the localization, activity and stability of Cdc24p.

PH domains consist of a C-terminal α -helix that sits below the core β -sandwich and three variable loop domains that interact with membrane phospholipids (171). The functional significance of PH domain recognition of phospholipids is currently unknown because mammalian PH domains (150) and *S. cerevisiae* PH domains (322) generally have both low specificity and affinity for specific phospholipids *in vitro*. Homo- or hetero-oligomer formation of proteins containing PH domains has been proposed as a mechanism to overcome low phospholipid affinity *in vivo*. 'Regulated avidity' (171) would result in multiple PH domains cooperating in the binding of phospholipids at the membrane that is temporally and spatially controlled.

Regulated avidity is potentially involved in the targeting and/or maintenance of Cdc24p at polarized growth sites through an interaction with Boi2p. Boi2p is a Bem1p-interacting homologue (22, 189) that localizes to incipient bud sites, the periphery of buds and the mother-bud neck in large-budded cells (113) and can be found in the nuclei of some G₁ cells (216). Boi2p was co-immunoprecipitated with Cdc24p and its polarized localization was dependent on Cdc28p-mediated phosphorylation at bud emergence (190). Overexpression of Boi2p results in the arrest of cells as large, round unbudded cells (22, 189). The carboxyl-terminal PH domain of Boi2p is necessary and sufficient for growth (22, 189) and mutation of this domain prevents localization to the bud (113). Taken together, this suggests that interaction between Cdc24p and Boi2p could result in targeting and maintenance at polarized growth sites through a PH-PH domain association (see **Chapter 3**).

PH domains can also function as an interface for protein-protein interaction. Heterotrimeric G-protein $\beta\gamma$ -subunits are recognized by some PH domains (299). This interaction occurs between the WD40 repeat of the β -subunit and the PH domain (309, 310). Membrane localization of a protein with a PH domain is likely achieved through a combination of phospholipids and protein recognition, each of which can be independently regulated, known as the 'dual-key strategy' (140). Dual recognition of the Cdc24p PH domain by phospholipids and recognition of additional amino acids located carboxyl-terminal to the PH domain (identified as the targeting domain (294), see below) by

regulatory protein(s) may be essential for the localization and maintenance of Cdc24p at sites of polarized growth since its PH domain bound strongly, but nonspecifically, to phosphoinositides and was not able to target to the membrane independently (170). ***Identification of the protein(s) that recognize this targeting domain was one focus of this dissertation research and is discussed in Chapter 3*** [see **Table 6** for proteins that interact with Cdc24p and **Targeting and Anchoring Domains** below].

The crystal structure of the DH-PH domains of mouse Dbs and human Cdc42 indicates that the PH domain interacts with the Switch II domain of Cdc42. Mutation of a highly conserved tyrosine (Y889) within the PH domain significantly decreases guanine nucleotide exchange (258). Since Cdc42 is membrane-bound via its carboxyl-terminal geranylgeranyl moiety, the Dbs PH-phospholipid and PH-Cdc42 interaction may assist with direction of the DH domain towards Cdc42 to facilitate guanine nucleotide exchange (PH-Cdc42 interaction). In addition this essential GEF activity could be spatially regulated via PH-phospholipid interactions (258).

PEST Sequences

The PEST motif proteolytic signal is composed of 12 or more amino acids enriched in Pro, Glu, Ser and Thr residues that are flanked by Lys, Arg or His residues (241). Cdc24p has two potential PEST sequences located within the PH domain (aa 490-504 and aa 535-555); however, given a PEST-FIND score of

less than +5 [(241),

<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=>

epestfind] they are unlikely to be of much significance. It is likely that other mechanisms are responsible for the decrease in Cdc24p levels at anaphase (293) [see **Conclusions and Future Directions**].

Ca²⁺-binding Domain

Cdc24p has two potential Ca²⁺-binding domains at its carboxyl terminus encompassing aa 649-658 (107, 198, 294) and aa 820-831 (107, 294). The amino acid sequences of both potential Ca²⁺-binding domains are illustrated in **Figure 5**. It was suggested that aa 649-658 binds Ca²⁺ in a manner analogous to α -lactalbumin (198); however, this seems unlikely given the fact that one prominent characteristic of this domain is that the Ca²⁺-binding loop is formed by a cysteine bridge (10), and there are no cysteine residues surrounding these amino acids. In further support of this, exogenously added Ca²⁺ does not alter GFP-Cdc24p localization during the cell cycle (294). However, mutations in this region (W659A and E653A, E654A, R656A) did result in a loss of GFP-Cdc24p targeting to sites of polarized growth (294), suggesting that these residues are interacting with protein(s) responsible for localization of Cdc24p specifically to sites of polarized growth (see **Chapter 3**).

Two α -helices connect a 12 amino acid loop in EF hand Ca²⁺-binding domains and positions 1, 3, 5, 7, 9, and 12 within this loop are responsible for ion

coordination (27). Residues within aa 820-831 that could be responsible for Ca^{2+} coordination are highlighted in bold. Mutation of two Asp residues (D824K and D831K) within this domain results in a loss of interaction with Bem1p (138). In conjunction with the fact that Ca^{2+} inhibits the *in vitro* interaction between Cdc24p and Bem1p (217), it is likely that this is a *bona fide* Ca^{2+} -binding domain. Ca^{2+} ion binding can alter protein conformation or facilitate interaction with negatively charged membrane phospholipids (60), and it is possible in this context that Ca^{2+} binding alters the conformation of the carboxyl-terminus of Cdc24p which prevents it from interacting with Bem1p. Given the fact that exogenous Ca^{2+} did not alter the localization of Cdc24p (294), it is possible that the Ca^{2+} ions lead to a release of $\text{PI}(4,5)\text{P}_2$ from Ca^{2+} -responsive proteins [i.e., calmodulin] (191), resulting in a localized pool of $\text{PI}(4,5)\text{P}_2$ that recruit polarity proteins with PH domains (i.e., Cdc24p and Boi2p).

Targeting and Anchoring Domains

The carboxyl-terminal 289 amino acids of Cdc24p were initially identified as necessary and sufficient for localization to polarized growth sites (293) [see **Figure 5**]. Further truncation of this region narrowed the targeting domain (TD) to 56 amino acids (647-704); however, stable maintenance of this region required the remainder of the carboxyl-terminus (aa 704-854) [anchoring domain] and interactions with Rsr1p, Bem1p and Tos2p (294). The Cdc24p single amino acid mutation W659A and the triple amino acid mutations E653A, E654A, R656A do

not target Cdc24p to sites of polarized growth and can't complement a *cdc24-4^{ts}* (294). Additionally, LG1C- (late-G₁ cyclin) mutants are unable to target Cdc24p to the incipient bud site (199), which results in bud emergence defects. This indicates that the ability to target Cdc24p to sites of polarized growth through protein-protein interactions, potentially mediated by phosphorylation, is essential.

Identification of proteins and potential phosphorylation sites within Cdc24p that function in the targeting and maintenance of Cdc24p at sites of polarized growth is discussed in Chapter 3.

p40^{phox} and Cdc24p (PC) Domain

PC domains [also referred to in the literature as OPR (octicosapeptide repeat), AID (atypical PKC-interaction domain) and OPCA (OPR, PC and AID)] are composed of conserved acidic and hydrophobic amino acids (210). The Cdc24p carboxyl-terminal PC domain (aa 780-854) binds to PB1 (p67^{phox} and Bem1p) (138). The structure of the Cdc24p PCCR (PC-motif containing region: aa 761-854) and the PB1 domain assumes a ubiquitin-like fold that facilitates their interaction (321). Heterodimerization of these domains is mediated by Bem1p basic residues [K480, K482 and R510] (138, 321) and acidic residues in the PCCR motif of Cdc24p [D820, D824, D831 and E832] (138, 291, 321). The interaction of Bem1p with Cdc24p via the PB1–PC domains plays a role in establishment and maintenance of cell polarity (34, 42, 106); however, the interaction is not essential unless Rsr1p or Tos2p are absent (294). This

suggests that in the absence of the Bem1p scaffold, targeting to Rsr1p or anchoring by Tos2p are sufficient to compensate for its function.

Cdc24p was suggested to be auto-inhibited through an interaction between the PC domain and a site between amino acids 673-780 that resembles a PB1 domain (277). This hypothesis was based on the observation that overexpression of *CDC24* lacking the PC domain was constitutively active if artificially targeted to the plasma membrane by myristylation, which could be suppressed by overexpression of the PC domain.

Cdc24p Potential Protein-Protein Interaction Partners

Table 6 illustrates proteins that interact directly with Cdc24p (yeast two-hybrid, genetic), are in a complex with Cdc24p (co-immunoprecipitation, predicted co-complex, genetic) or bind to DNA sequences that regulate expression of *CDC24* (DNA cross-linked). **Table 7** lists potential Cdc24p domains identified by ELM analysis, in conjunction with SGD and literature searches, to determine potential *S. cerevisiae* ligands.

The interactions presented in **Table 6** were determined by high-throughput (HTP) methods or through curation of published literature. The numbers of interactions determined by each method are equivalent; however, the overlap between each method is only 14% (243). Yeast-two hybrid HTP assays are known to generate a large number of false-positives and less than 3% have been validated by other methods (243).

Table 6: Potential interaction partners of Cdc24p.

This table combines all of the interaction data for Cdc24p currently available (as of 02/2008) from SGD, Yeast Resource Center, BioGrid (36, 285), BIND (6), BioPixie (208), DIP (320) and MIPS Protein Interaction and Complex Database.

Identification Method	Proteins Identified
Yeast two-hybrid	BE/M: Bem1, Far1, Rsr1, Cdc42, Gic2, Bem4, Sec15, Ste20, Swe1, Tos2, Cla4, Boi2 CAPs: Ent2, Rvs161, Hua1, Akr1 mRNA: Air1, She2 Chaperonin: Cct8 ER: Lip1 DNA: Rsc2, Soh1
Co-Immunoprecipitation	BE/M: Axl2, Boi1, Rga2 Chaperone: Sis1, Hsp42 Ribosome protein: Rpl17b, Rpl4b, Rpp0
Predicted co-complex	BE/M: Bud5, Gic1, Gic2, Rho1, Cdc42, Rsr1, Bem1, Bni1, Tpm2, Rom2 HOG: Sho1 RAM: Mob2 Septum: Skt5, Lrg1
Genetic	BE/M: Rdi1, Msb1-4, Gic1, Sec15, Bem2 Vacuole: Cep3, Cse4, Rtf1, Csl4, Vma5 MEN: Lte1 Vesicle transport: Sbe2, Bmh1

Functional classification of interacting proteins:

BE/M: bud emergence and/or maintenance

CAPs: cortical actin patches; endocytosis

mRNA: associated with mRNA transport; **ER:** endoplasmic reticulum-associated

HOG: high-osmolarity glycerol MAPK pathway

RAM: regulation of ACE2p activity and morphogenesis network

Septum: protein involved in septum formation

MEN: mitotic exit network

Proteins cross-linked with predicted DNA regulatory regions of CDC24:

Sir3, Sir4, Abf1, Tbp1, Mlp1, Nup116

The majority of Cdc24p interaction data was obtained from HTP screens – affinity-capture followed by mass spectrometry (89, 90) and yeast two-hybrid (75). If just the data obtained from these are examined, 7 proteins were isolated by affinity-capture in a complex with Cdc24p and 11 were identified to directly interact by two-hybrid. Only one protein is identified by both methods – Bem1p. If the other proteins identified by individual experiments are included, the overlap between affinity-capture (16 proteins identified) and two-hybrid (14 proteins identified) is only 5 proteins (Bem1p, Far1p, Ste4p, Cdc42p and Rsr1p), or 16.7% overlap. MIPS (Munich Information Center for Protein Sequences) Protein Interaction and Sequence Database does not have HTP data (193) and has therefore been called the ‘gold standard’ for bioinformatics research (162).

With no preconceived notions about the accuracy of this information, deletion strains of many of the proteins listed in **Table 6** were screened for their ability to localize the targeting and anchoring domain (aa 647-854) of Cdc24p to sites of polarized growth. ***Deletion strains unable to localize GFP-Cdc24 (aa647-854)p identified proteins that play a role in targeting and maintenance of Cdc24p at sites of polarized growth*** (see **Chapter 3**). The information presented in **Table 7** provides information that will be essential for additional analysis of the mechanisms that target and maintain Cdc24p at sites of polarized growth.

Table 7: Eukaryotic Linear Motif (ELM) Server predictions for Cdc24p.

Cdc24p is divided into four primary domains in this table: the NH₂-terminus, CH and PH domains and targeting and anchoring domain (aa 647-854). The ELM motifs predicted for each domain are listed, with a brief description of each motif. The numbers following the motif in () indicate the number of motifs within this domain (1 motif identified if no number is present). Proteins that could interact with these motifs based on their function are listed in the last column.

Domain	Motif	Potential Interaction(s)
NH ₂ -terminus	FHA ligand: phosphr-Thr recognition site for FHA domains found in nuclear proteins APC/C ligand (2): anaphase-promoting Ub-ligase destruction box motifs Cyclin ligand: cyclin recognition site SH3 ligand: recognition site for proteins with SH3 domains PIKK: phosphoinositide-3-OH-kinase-related kinase consensus site 14-3-3 ligand: phospho-Ser/Thr recognition Clathrin box: motif present in cargo adaptor proteins CK1 (7): casein kinase 1 consensus sites GSK3 (11): glycogen synthase kinase consensus sites PKA (3): cAMP-dependent protein kinase A consensus sites	Dma1-2p, Xrs2p, Vps64p Apc1-2p, Cdc16p Cln1-3p, Clb1-6p Boi1-2p, Rvs167p, Bem1p Lsb6p, Tor1-2p Bmh1p, Bmh2p Chc1p Yck1p Mrk1p, Ygk3p Tpk1p
CH	PLK: Polo-like kinase Endocytic targeting: Tyr-based sorting signal PIKK (4) GSK3 (3) SH3 ligand FHA ligand (2) Cyclin ligand BRCA1 ligand (2): phospho-Ser recognition CK1 ProDKin: proline directed kinase	Cdc5p Ent1p, Ent2p, Apl2p Lsb6p, Tor1-2p Mrk1p, Ygk3p Boi1-2p, Rvs167p, Bem1p Dma1-2p, Xrs2p, Vps64p Cln1-3p, Clb YHL010cp Yck1p CDKs and MAPKs

Domain	Motif	Potential Interaction(s)
PH	PP1: protein phosphatase 1 binding motif Endocytic targeting	Glc7p Ent1p, Ent2p, Apl2p
aa 647-854	14-3-3 ligand BRCA1 ligand (2) WW (2): phospho-Ser dependent recognition CK1 (9) CK2 (3): casein kinase 2 consensus site GSK3 (15) PKA (4) ProDKin (2)	Bmh1p, Bmh2p YHL010cp Rsp5p, Ess1p Yck1p Yck2p Mrk1p, Ygk3p Tpk1p CDKs and MAPKs

The information presented in this table was compiled from the Eukaryotic Linear Motif (ELM) server (<http://www.elm.eu.org/>). The amino acid sequence of Cdc24p was entered, and the server predicted functional sites based on short, linear subsequences that have been related to an interaction with another protein, and uses a set of logical filters to remove false positives.

*The information presented in this table can be used in the future to broaden our understanding of how Cdc24p activity and subcellular localization affect the subsequent activation of Cdc42p (see **Conclusions and Future Directions**).*

Localization of GFP-Cdc24-D824K, D831Kp and GFP-Cdc24-647-854^{D824K, D831K}p is comparable to GFP-Cdc24p in a wild-type strain, suggesting that protein(s) in addition to Bem1p are involved in the maintenance of Cdc24p at sites of polarized growth (294). *rsr1Δ*, *bem4Δ*, *tos2Δ* and *cla4Δ* mutants exhibited wild-type targeting of GFP-Cdc24-647-854p. Targeting of GFP-Cdc24-647-854^{D824K, D831K}p in *rsr1Δ* and *tos2Δ* mutants is significantly abrogated (<5% of wild-type) to incipient bud sites and mother-bud necks, and additionally in *tos2Δ* mutants at tips of small buds. This suggests that Bem1p acts in conjunction with other proteins to target and maintain Cdc24p at sites of polarized growth. GFP-Cdc24-647-854^{D824K, D831K}p and GFP-Cdc24-647-854p fractionates primarily in the pellet. In a *tos2Δ* mutant, GFP-Cdc24-647-854^{D824K, D831K}p and GFP-Cdc24-647-854p fractionates equivalently in the pellet and supernatant, suggesting that Cdc24p is maintained at the plasma membrane at sites of polarized growth, in part, via an interaction with the transmembrane protein Tos2p.

SKG6 shares 35% sequence homology with *TOS2*, most of which is within their carboxyl-termini. SKG6 was originally identified as a dosage suppressor of the cell wall defects of a *kex2Δ gas1Δ* mutant (297) and of *cdc42-118* (75). *TOS2* is unable to suppress the cell wall defects of a *kex2Δ gas1Δ* mutant, suggesting that they are not functionally redundant in this regard. SKG6 and *TOS2* have also been identified as dosage suppressors of a *gic1 gic2-Ts* mutant and

overexpression of either Skg6p or Tos2p resulted in cells with septum, cytokinesis and septin localization defects (86). These results suggest that while Skg6p and Tos2p have divergent roles in cell wall maintenance, they may have conserved roles in polarity maintenance (see **Chapter 3**). Taken together, these results suggest additional, unidentified proteins also participate in the targeting and maintenance of Cdc24p and formed the basis for the screen discussed in **Chapter 3**.

The GTPase Activating Proteins (GAPs) Rga1p, Rga2p, Bem2p and Bem3p

Rga1p, Rga2p, Bem2p and Bem3p are members of the RhoGAP family, characterized by a conserved 150 aa RhoGAP domain (201). Overexpression of GAP-deficient Rga1p and Rga2p (47, 282) and Bem3p (153) results in hyperpolarization of buds. Overexpression of Bem3p arrests cells as unbudded cells with a polarized actin cytoskeleton, and *bem2Δ* mutants target Cdc24p to multiple cortical sites (153). These morphological phenotypes indicate that the GAPs are negative regulators of the central regulator of polarity, Cdc42p. Because the GAPs are not a focus of this dissertation, they will not be discussed as extensively as Rdi1p (GDI) and Cdc24p (GEF) and will be mentioned only when they have been shown to have a direct role in establishment and maintenance of cell polarity in the context of GDI and GEF interactions (see **GTPase Structure and Interaction with Regulators and Effectors**).

Establishing Cell Polarity at the Incipient Bud Site

The cellular components that either localize initially to the incipient bud, are maintained at the bud tip and then are retargeted to the mother-bud neck before cytokinesis have been termed the 'polarity cap network' (204). Establishment of this network at the incipient bud site occurs in wild type, haploid *S. cerevisiae* through recognition of an axial landmark, or spontaneously via stabilization of components at a discrete cortical site in mutants lacking components of the landmark (196).

Cortical Landmark

'Landmark' proteins remain at the bud site (mother-bud neck) generated in the previous cell cycle to direct the formation of the new axial bud site in haploid yeast. The axial landmark consists of the septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p) (82), Bud3p (51), Bud4p (50, 266), Axl1p and Axl2p (114, 255). Loss of any of these proteins results in loss of axial budding and results in a bipolar budding pattern.

A septin ring forms at the incipient bud site in late G₁ ~15 minutes before bud emergence, transforms into a collar when the bud emerges that remains at the mother-bud neck throughout bud growth, and then splits into two rings (mother and bud) during cytokinesis (97). The previous septin ring and associated landmark proteins disassemble as the new ring is formed. The GAP Rga1p prevents formation of a new bud at the previous bud site by keeping

Cdc42p in the inactive GDP-bound state (298). Cdc42p, its effector proteins Gic1p and Gic2p (142) and Cdc24p (142, 144) are required to recruit septins to the incipient bud site, which implies that GTP-bound Cdc42p is involved in the recruitment.

The septins can therefore define three cortical domains: mother, bud and neck. A detailed localization of 40 proteins that localize to the neck cortex, and their specific localization within these cortical domains can be found in Gladfelter, *et al.* (97). The functions of Bud3p and Bud4p are unknown. The only recognizable domain present in each protein is a DH and PH domain, respectively. Axl2p (also referred to as Bud10p in the literature) is a single-pass transmembrane protein with cadherin-like domains in its 500 extracellular amino acids. It has been proposed that Axl2p is anchored at this site by interaction of the cell wall with its glycosylated extracellular domain and that Bud3p and Bud4p interact with the intracellular domain to facilitate clustering of the bud site selection 'receptor' (49).

If cells are arrested in G₁, the landmark is not stably maintained and buds are formed at a random site upon release (52). The landmark proteins can therefore be considered a non-essential initial asymmetry. Competition for other sites on the membrane is unlikely to occur, since activators will be recruited specifically to this site; however, in their absence a random site on the membrane will be chosen stochastically and become the site of polarization (100) [see **Feedback Loops Stabilize Asymmetry and Polarize the Cytoskeleton**].

Polarity Establishment Proteins Recognize the Landmark

The cortical landmark is recognized by the GAP Bud2p and the GEF Bud5p, which spatially activate the uniformly plasma membrane-localized Ras GTPase Rsr1p (also referred to as Bud1p in the literature) (50, 51). Cdc24p interacts with and stabilizes GTP-bound Rsr1p to ensure that Cdc24p is spatially restricted to the incipient bud site (147, 229, 230, 329). When Rsr1p is GDP-bound, it interacts with Bem1p (229) which may facilitate the interaction between Cdc42p and Cdc24p (159). Cdc42p, Cdc24p and Bem1p were co-immunoprecipitated with Axl2p (87), suggesting that these proteins could directly interact with the landmark. Sequestering the GEF Cdc24p to this site, as described previously for Rsr1p, prevents the uniformly plasma membrane-localized Rho GTPase Cdc42p from becoming active at any site but the incipient bud site.

Feedback Loops Stabilize Asymmetry and Polarize the Cytoskeleton

In *S. cerevisiae*, *bem1-8 rsr1Δ* mutants are unable to polarize Cdc42p at the restrictive temperature, indicating that either the Bem1p scaffold (positive feedback loop formation) or the recognition of the spatial landmark (Rsr1p) is required to stabilize asymmetric localization of Cdc42p (135). However, this is an oversimplified view of the requirements for the asymmetric localization and

maintenance of polarity proteins [see **Maintaining Polarized Growth at the Bud Tip**].

‘Symmetry breaking’ begins in *S. cerevisiae* with an actin-independent positive feedback loop involving Cdc42p activation by Cdc24p that is facilitated by Bem1p (135), which is followed by an actin-dependent phase that reinforces the initial asymmetry through delivery of GAPs (225). This may seem counter-intuitive, but makes sense in light of the cell polarity requirement for GTP-GDP cycling by Cdc42p (135). The GTP-hydrolysis requirement has been proposed to be essential because it permits the continuous interaction of GDP-bound Cdc42p with Cdc24p, which could be important for effector protein localization and/or targeting (135) [see **Chapter 4: The Rho GEF Cdc24p as a Signal Integrator and Scaffold**]. This analysis makes sense because although the only function currently ascribed to Cdc24p is GDP-GTP exchange for Cdc42p, the catalytic DH domain only comprises approximately 20% of the protein. The remaining 80% of the protein contains multiple ligand motifs for interaction with a wide range of proteins (see **Table 7**).

Maintaining Polarized Growth at the Bud Tip

Approximately 50 proteins (SGD GO term annotation of ‘cellular bud tip’ as of 01/2008, excluding proteins that function in the mating pathway) are known to localize to the bud tip and have roles in: bud site selection and establishment of polarity, exocyst and polarisome components, cell wall integrity signaling and

remodeling and MAPK signaling (137). Eukaryotic lipid rafts are plasma membrane microdomains enriched in sterols and sphingolipids that have essential functions in sorting and sequestering signal transduction proteins and in maintenance of cell polarity (8). In yeast these sterol-rich domains (SRDs) are evident at the tips of mating projections in *S. cerevisiae* and the tips of hyphae in *C. albicans*, but they are not present during budded growth in *S. cerevisiae* (8); therefore, in contrast to other eukaryotes, they have no role in maintenance of polarized growth and will not be discussed.

The Cdc42p 'polarity cap' of incipient and small budded cells does not appear to be identical beyond their composition. The caps of budded cells were more resistant to dispersal of GFP-Cdc42p than were the caps at incipient bud sites following disruption of actin cables [cortical actin patches were not effected](136). This indicates that additional mechanisms of maintenance of the polarized cortical membrane domain (see **Figure 6**) are present after bud emergence. Potential mechanisms are discussed in the following section: diffusion restriction, phosphoinositide generation, targeted vesicle, mRNA and cER localization and endocytosis followed by redelivery to the plasma membrane.

Figure 6: Factors that contribute to maintenance of the polarized cortical domain.

The five factors illustrated here have a role in the maintenance of bud tip polarization. Proteins that interact with Cdc42p (**green**), Cdc24p (**blue**) or both proteins (**orange**) are highlighted. Cdc24p-interacting proteins could facilitate the initial delivery of Cdc24p to the incipient bud site and its continued maintenance at bud tips (see **Chapter 3** and **Discussion**).

Maintenance of Polarization

PI Localization

Mss4p, Lem3p, Dnf1p, Dnf2p

Diffusion Restriction

Bem1p, Tos2p, Boi2p, Boi1p, Septins, Axl2p

Vesicle Transport

Myo2p, Bmh1p, Bni1p, Sec15p, Sbe2p

mRNA and cER Localization

ASH1, CDC42, She2p, She3p, She4p, Bni1p, Myo4p, Bud6p, Air1p, Yck1p, Khd1p, Puf6p

Endocytic Recycling

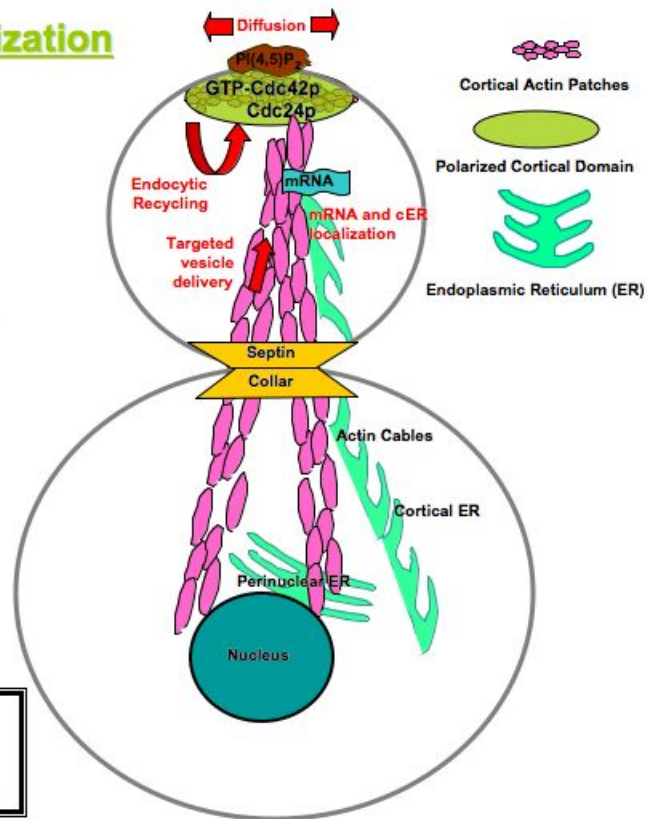
Vps54p, Ent2p, Rvs161p

Interacts with:

Cdc24p

Cdc42p

Cdc24p and Cdc42p



Establishing Polarized Cortical Domains:

The Plasma Membrane Phosphoinositide Signaling Hub

The primary phospholipid components of the plasma membrane in *S. cerevisiae* are phosphatidylethanolamine (PE), phosphoinositol (PI), phosphatidylserine (PS), inositol sphingolipids and sphingomyelin (SM) (303). Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], which constitutes only 1% of plasma membrane lipids in eukaryotic cells, anchors proteins at discrete sites on the plasma membrane, activates ion channels, is required for endocytosis and exocytosis, regulates actin-binding proteins, and produces the second messengers PI 1,4,5-trisphosphate [PI(1,4,5)P₃], PI(3,4,5)P₃ and diacylglycerol (DAG) (reviewed in (191)). PI(4,5)P₂ and PI(3,4,5)P₃ are proposed to be 'signaling hubs' because of their roles in targeting GTPases with C-terminal polybasic regions [i.e., Cdc42 and Rho] (118).

Phosphoinositide Regulation

In *S. cerevisiae*, Stt4p generates PI(4)P at the plasma membrane, which is then converted to PI(4,5)P₂ by Mss4p (15). Mss4p undergoes nuclear-cytoplasmic shuttling to control its activity and must be phosphorylated by Yck1p at the plasma membrane for stable membrane maintenance (14). Arf3p (ADP-ribosylation factor), its GEF Yel1p and GAP Gts1p regulate the levels of PI(4,5)P₂ at sites of polarized growth (281). In cells arrested in G₁, PI(4,5)P₂ is uniformly

distributed around the plasma membrane (311), suggesting that a G₁-dependent signaling event results in PI(4,5)P₂ and PI(3,4,5)P₃ production at sites of polarized growth to direct targeting and maintenance of polarity proteins, including Boi2p, which is synthetic lethal in a *mss4^{ts}* mutant (16).

PE is translocated to the outer leaflet at incipient bud sites and the tips of small buds by the P4-ATPases Dnf1p and Dnf2p and their regulatory subunit Lem3p (263). An inability to translocate PE back to the inner leaflet results in maintenance of Cdc42p and Boi1p, polarisome and exocyst components, but not Cdc24p, at the tips of large buds post-anaphase (263). This indicates that retargeting of Cdc24p to the mother-bud neck post-anaphase is independent of this process.

The GEFs Rom1p and Rom2p are present at the incipient bud site and the tip of small-budded cells (185, 222) and are believed to be responsible for the translocation of Rho1p from a soluble pool maintained by Rdi1p. Rom2p activation of Rho1p and Rho2p is dependent on the Tor2p lipid kinase (268). This suggests generation of phosphoinositides that interact with the PH domain could play a role in localization and activation of other Rho GTPases via GEF translocation.

An *in vitro* assay demonstrated that the GAP activity of Rga1p and Rga2p towards Cdc42p was stimulated by PE and PS but was inhibited in the presence of PI(4,5)P₂ (263). Additionally, polarized localization of Gic2p requires an intact CRIB domain and an amino-terminal polybasic region that interacts with

PI(4,5)P₂ (220). Together, this suggests a mechanism by which positive regulators and effectors of Cdc42p could be localized and activated at polarized growth sites by PI(4,5)P₂ and negative regulators by PE and PS, which would be inactivated by PI(4,5)P₂.

Protein Domains Recognize Phosphoinositides

Proteins with BAR (Bin, amphiphysin, Rvs) and ENTH (epsin N-terminal homology) domains participate in endocytosis. The BAR and ENTH domains form an amphipathic helix when they bind to acidic phospholipids [Figure 7] in the plasma membrane, which results in curvature of the membrane, facilitating endocytosis (reviewed in (139)). F-BAR [FCH (FER/CIP4-homology) domain with a C-terminal coiled-coil domain] domains are often found in proteins functioning in cytokinesis and actin polymerization. These proteins translocate from the cytoplasm to the plasma membrane, can bind transmembrane proteins to facilitate clathrin-mediated endocytosis, and contain additional motifs for GTPase and phosphoinositide interaction. ***S. cerevisiae proteins with an F-BAR domain (Boi2p) and an ENTH domain (Ent2p) (see Table 8) have a role in localization and/or maintenance of Cdc24p at sites of polarized growth (see Chapter 3).***

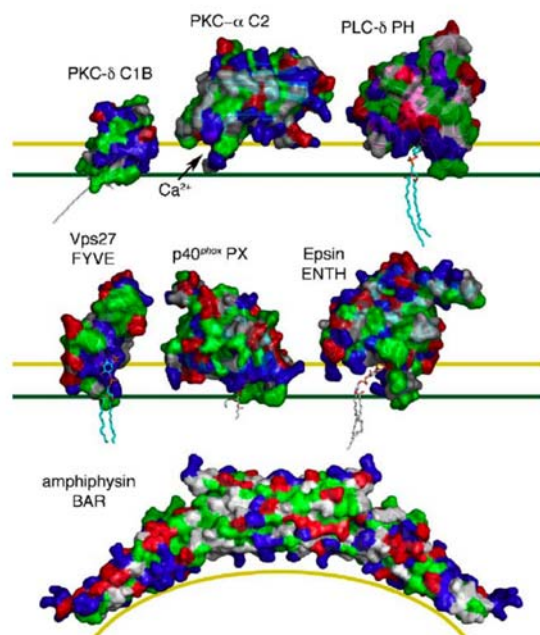


Figure 7: Membrane-binding domains.

Molecular surface models of common membrane-binding domains reproduced from (133). The gold line represents the top of the polar membrane bilayer and the green line the hydrocarbon core.

Table 8: Phosphoinositide-binding domains in *S. cerevisiae*.

Domain: protein domains that are known to bind phospholipids; **PI(4,5)P₂:** yes – the domain only recognizes PI(4,5)P₂, no – the domain recognizes other phosphoinositides in addition to PI(4,5)P₂; **# of proteins** in Sc with this domain; **representative proteins** are proteins with this domain that are discussed in this dissertation. The information presented in this table was compiled from SGD, (307).

Domain	PI(4,5)P ₂ only	# of Proteins	Representative Proteins
ENTH ANTH	No	15	Ent1p, Ent2p, Sla2p
C2	Yes	11	Pkc1p, Rsp5p, Bud2p
PH	No	38	Cdc24p, Boi1p, Boi2p, Cla4p, Bem2p, Bem3p
PX	No	15	Bem1p
BAR	No	7	Rvs167p, Boi1p, Boi2p

ENTH epsin N-terminal homology; **ANTH** AP180 N-terminal homology; **C2** lipid binding; **PH** Pleckstrin homology; **PX** phox homology; **BAR** Bin, amphiphysin, Rvs

Establishing Polarized Cortical Domains:

mRNA and cER Localization

Trafficking of mRNA to, and translation at, sites of polarized growth is essential for the maintenance of polarity across fungal species (325). Translation at sites of polarized growth is facilitated by cortical endoplasmic reticulum (cER), which is transported to the tip of the growing bud by the actin cytoskeleton [reviewed in (306)]. cER is anchored at incipient bud sites and the tips of buds through S phase and into early G₂ phase (81) by the integral ER protein Scs2p (179) and Shs1p, a nonessential septin (182). mRNA targeting to the bud tip and septin diffusion restriction [see **Diffusion Restriction**] work together to ensure asymmetric protein localization between mother and bud (289).

ASH1 is the most extensively studied bud-localized mRNA. Ash1p is responsible for repressing *a/α* mating-type switching in daughter cell nuclei (28). Asymmetric localization of *ASH1* to the bud tip is dependent on the myosin motor Myo4p, the mRNA-binding protein She2p and the mRNA-Myo4p adapter protein She3p (99). Polarisome components Bni1p and Bud6p (21) and the Myo4p-She2p-She3p 'locasome' complex (98) are required for anchoring of transcripts at the bud tip. Surprisingly, it is the secondary structure of the *ASH1* coding sequence, not the 3' UTR, that is the essential zipcode for localization (99). The motifs that She2p recognizes in the *ASH1* E1, E2A and E2B stem-loops were identified and are conserved in two other bud-localized transcripts (218). This suggests this motif might be shared with other bud-localized transcripts targeted

by She2p (i.e., Cdc42p, see below, and Cdc24p, and other proteins listed in **Figure 6**).

In *S. cerevisiae*, twenty-two bud-localized mRNAs were identified, and of these, 41% encoded membrane-associated proteins (273). This suggests that mRNA and cER trafficking to bud tips might be important to ensure that proteins lacking membrane-anchoring modifications can be specifically targeted to sites of polarized growth (12); however, in *she2Δ* mutants polarized localization of proteins whose mRNA is normally trafficked was not effected (273). Although mRNA trafficking may be essential for daughter-specific proteins (i.e., Ash1p), it is apparent that these proteins also contain intrinsic targeting information. The targeting of Cdc24p to sites of polarized growth was examined in a *she2Δ* mutant (see **Chapter 3**). Cdc24p exhibits wild-type targeting in the *she2Δ* mutant, suggesting that either *CDC24* is not targeted to bud tips, or that Cdc24p achieves polarized localization with or without She2p-dependent targeting.

70-80% of pre-anaphase, small-budded cells have *CDC42*, *SEC3*, *EXO84*, and *RHO3* mRNA targeted to the bud tip (13). Targeting of *CDC42* mRNA and Cdc42p cytoplasmic localization occurs prior to bud emergence (13), indicating that this is one potential mechanism to ensure a pool of GDP-bound Cdc42p that can be rapidly translocated to the incipient bud site. Furthermore, PI(4)P generation by the PI 4-kinase Stt4p [see **The Plasma Membrane Phosphoinositide Signaling Hub**] is required for translation initiation (45);

therefore, generation of PI(4)P at sites of polarized growth can initiate translation of targeted mRNA.

Maintaining Polarized Cortical Domains:

Targeted Vesicle Delivery

There are four essential steps in vesicle transport: [1] budding from donor compartment, [2] transport towards the acceptor compartment, [3] tethering to the acceptor compartment, and [4] fusion with the acceptor membrane (33). Functional and regulatory proteins participate at each step to ensure fidelity from the donor to acceptor membrane [reviewed in (44)]. Clathrin or coat protein complexes (COPI and II) are recruited to membranes by members of the Arf-family of GTPases, which facilitate vesicle budding from donor membranes. The vesicle reaches its destination via diffusion or directed transport by myosin motors along the actin cytoskeleton; therefore, mutation of proteins that nucleate actin assembly (*i.e.*, formins) or affect its stability (*i.e.*, tropomyosin) adversely affect cell polarity [reviewed in (204)].

Spatial restriction of exocytosis is essential for polarized cell growth and morphogenesis. The exocyst is composed of eight primary components: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p; these components tether post-Golgi secretory vesicles to assist SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors)-mediated membrane fusion at sites of polarized growth [reviewed in (207)]. Sec3p, the spatial landmark that directs vesicle targeting and cER inheritance (315) [see **mRNA and cER Localization**],

localization to these sites is dependent on interaction with Cdc42p (327) and Rho1p (108). Spatial regulation of secretion can be coordinated with polarization through the interaction of the bud-emergence scaffold Bem1p with the exocyst component Sec15p (84, 323). Localization of the exocyst components Exo70p (116) and Sec3p (326) are dependent on the interaction of their poly-basic regions with PI(4,5)P₂ at sites of polarized growth, as is the localization of Cdc42p and its regulatory and effector proteins [see **The Plasma Membrane Phosphoinositide Signaling Hub**].

FRAP (Fluorescence Recovery After Photobleaching) studies indicate that the three components involved in actin-independent symmetry breaking (Cdc42p, Cdc24p and Bem1p) (135) [see **Feedback Loops Stabilize Asymmetry and Polarize the Cytoskeleton**] are dynamic, with a half-time of recovery to the plasma membrane of ~ 5 seconds for Cdc42p and Cdc24p and ~3 seconds for Bem1p (312). Since Bem1p only recovered to ~60% of the pre-bleach level, Bem1p could be the limiting partner for association between Cdc42p and Cdc24p.

Cdc42-Q61Lp (GTP-bound) and Cdc42-D57Yp (GDP-bound) have a half-time of recovery to the plasma membrane of ~60 seconds and ~40 seconds, respectively (312). 52% of wild-type Cdc42p was cytosolic, while <10% of the constitutively GTP- or GDP-bound Cdc42p was cytosolic, indicating that Cdc42p must exchange nucleotide to be removed from membranes. Additionally, if Cdc42p is locked in either state, delivery to bud sites becomes dependent on the

secretory pathway. It has been suggested that Msb3p maintains a pool of cytosolic GDP-bound Cdc42p at the bud tip (290). All experimental observations together suggest a model in which the pool of GDP-bound Cdc42p maintained at polarized growth sites is depleted to initially establish clustering of Cdc42p at the polarized cortical domain that is then activated by Cdc24p. GTP-bound Cdc42p interaction with effector proteins then target vesicle delivery to the site, which would replace the pool of GDP-Cdc42p. After polarity establishment, the *S. cerevisiae* OSBP (oxysterol-binding protein) homologue Osh4p is involved in the maintenance of Cdc42p and Rho1p at the tips of growing buds by regulating targeted vesicle delivery of either the GTPases or their regulators (158). This represents one potential mechanism by which the cytoplasmic pool of Cdc42p can be replaced and regulated. Cdc24p has genetic interactions with several proteins involved in vesicle transport; however, we did not assay targeting of Cdc24p in vesicle transport deletion mutants (see **Chapter 3**), so any potential role they may have in targeting or maintenance of Cdc24p is unknown.

Maintenance of Polarized Cortical Domains:

Diffusion Restriction

Lateral diffusion of proteins and phospholipids within the plasma membrane can be restricted by a 'membrane-skeleton fence' that interacts with 'anchored transmembrane protein pickets' (288), and by oligomer formation, which counteracts the effects of single molecule diffusion through the 'pickets' (288) [see **Figure 8**]. The picket-fence interaction can create a short-term,

Figure 8: The Picket and Fence Model.

Immobilized transmembrane protein 'pickets' (blue and yellow) that are immobilized through association with the membrane skeleton 'fence' prevent the free diffusion (red and green lines within the black box) of associated peripheral membrane proteins (orange). Image adapted from (209).

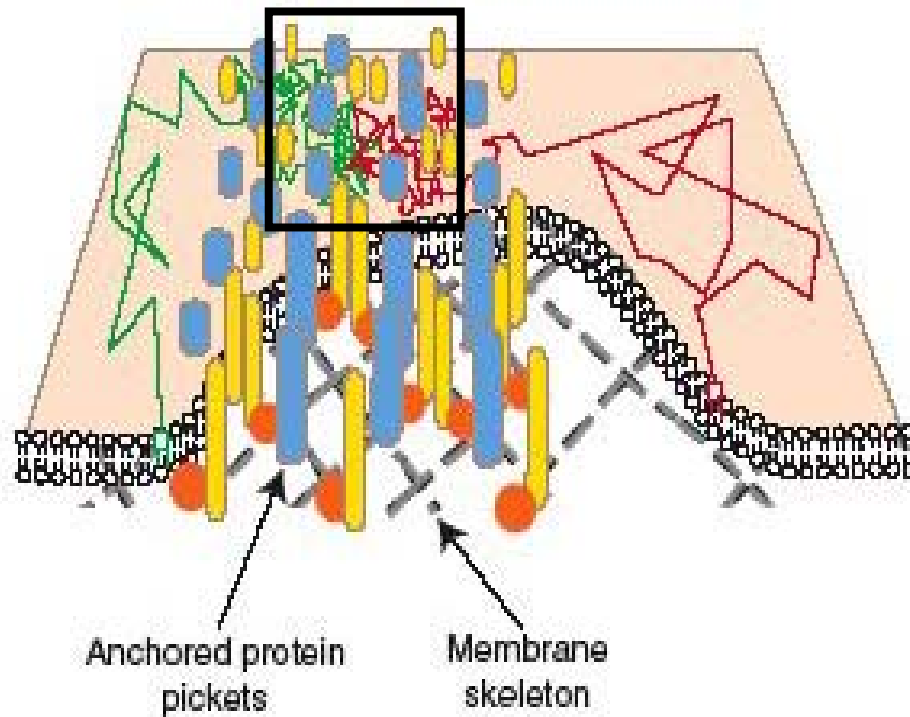
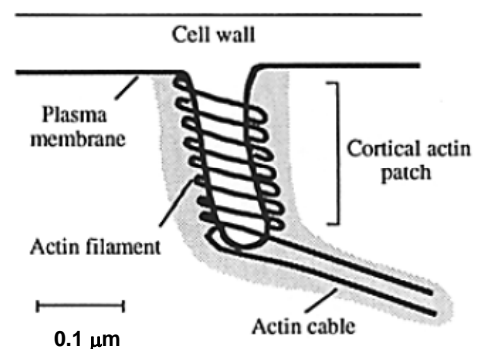


Figure 9: Cortical actin patches connect the actin cytoskeleton to the plasma membrane.

Cortical actin patches, cytoskeleton-associated plasma membrane invaginations [image adapted from (205)], which are localized at sites of polarized growth could, in addition to transmembrane proteins, act as anchored pickets to prevent the free diffusion of peripheral membrane proteins essential for the establishment and maintenance of polarized growth.



confined plasma membrane compartment. Hop diffusion will occur long-term when a protein ‘hops’ the fence if it encounters a space between the cytoskeleton and plasma membrane (see **Figure 8**, red and green lines outside of the black box represent hop diffusion).

S. cerevisiae transmembrane proteins mentioned thus far that could participate in the restriction of peripheral membrane proteins (*i.e.*, Cdc24p, Bem1p, and Boi2p) at sites of polarized growth are Axl2p and Tos2p. Axl2p can be co-immunoprecipitated with Cdc42p, Cdc24p and Bem1p (87), indicating that they could be associated in a large protein complex. Tos2p is known to play a role in anchoring of Cdc24p to bud tips (294) and Cdc24p has been shown to form dimers, which was also important for bud tip localization (195). Additionally, Cdc24p interacts with Ent2p, Rvs161p, Hua1p and Akr1p (see **Table 6**), all components of cortical actin patches (see **Figure 9**). Cortical actin patches connect the plasma membrane to the actin cytoskeleton (205), form a ring around the incipient bud site and are associated with other sites of polarized growth (9) where they may, in addition to acting in endocytosis, prevent diffusion of peripheral membrane proteins away from sites of polarized growth.

The *S. cerevisiae* septins expressed during vegetative growth (Cdc3p, Cdc10p, Cdc11p, Cdc12p and Shs1p) are GTP-binding proteins with roles in bud-site selection (82), asymmetric protein localization (20, 161, 289), spindle orientation, and the morphogenesis checkpoint [reviewed in (181)]. The septin collar has no apparent mother-bud polarity (308); therefore, asymmetric

localization of proteins must be directed by other mechanisms (phosphoinositides, vesicle, mRNA and cER delivery to the bud), while the septins function as a general diffusion barrier to separate mother from bud.

Maintenance of Polarized Cortical Domains:

Endocytic Recycling

Targeted vesicle delivery and localized protein synthesis by targeting of mRNA and cER are the primary mechanisms for initial asymmetric protein localization and maintenance at the plasma membrane in yeast. Restricting the diffusion away from this polarized cortical domain, or endocytosis and subsequent recycling back to the plasma membrane (endocytic recycling) can then maintain asymmetric protein localization.

Ubiquitin (Ub)-dependent endocytosis of the plasma membrane proteins Ste6p, Ste2p and Ste3p results in their targeting to the yeast vacuole for degradation [reviewed in (120)], while Ub-independent endocytosis of Ste3p and Ste6p results in recycling back to the plasma membrane (54, 163). The latter form of endocytosis was found to be mediated by NPF $X_{(1,2)}$ D (X represents any amino acid) motifs that additionally function in polarized localization of the cell wall stress sensor Wsc1p (234) the chitin synthase Chs3p (57), and the P4-ATPases Drs2p and Dnf1p (178).

Several components of this endocytic recycling pathway have been identified in yeast: Rcy1p, Skp1p, Tlg1p and Tlg2p [reviewed in (128)], Ypt31p, Cdc50p, Snc1p and Drs2p (85), retromer components (Vps35p, Vps29p,

Vps26p, Vps5p and Vps17p) [reviewed in (271)], and Gyp1p (165). A mathematical model for the cortical localization of polarized proteins (186) predicts that Cdc42p and its effectors, but not its regulators, can maintain polarity through a combination of slow membrane diffusion (302), endocytosis from the center of the polarized domain, and subsequent recycling back to this domain. Cdc42p and its effector proteins are not known to interact with the three cargo selector proteins of the retromer (Vps35p, Vps29p and Vps26p), so the significance of their endocytic recycling to sites of polarized growth is currently unknown.

Redirecting Polarized Growth to the Mother-Bud Neck

The cellular components that localize initially to the incipient bud, are maintained at the bud tip and then are retargeted to the mother-bud neck before cytokinesis have been termed the 'polarity cap network' (204). This includes the exocyst and polarisome complexes, the axial landmark proteins, cortical actin patches, cell wall synthesis enzymes, and central to this dissertation, Cdc42p and its regulators Cdc24p and Rdi1p. Additionally, the septins, which have remained at the mother-bud neck since their assembly in G₁, serve as a scaffold to assemble other necessary cytokinesis components throughout the cell cycle (*i.e.*, Myo1p and Iqg1p). Therefore, it is during G₁ that the future axis of division (mother-bud neck) is established.

Cytokinesis and septum formation must be spatially and temporally regulated to ensure that a full chromosome and organelle complement is

delivered to the bud and that the cell wall is completely formed before cell separation [reviewed in (43)]. A series of cell cycle checkpoints exists in *S. cerevisiae* to monitor the progress of each event.

The mitotic exit network (MEN) is the cell cycle checkpoint that determines whether the spindle is oriented correctly in anaphase and permits degradation of the mitotic cyclins by promoting release of the Cdc14p phosphatase from the nucleolus [reviewed in (279)]. MEN is driven by a GTPase signaling cascade controlled by Tem1p (19). Tem1p localizes to the spindle pole body that is transported into the bud. Its bud-tethered GEF Lte1p activates Tem1p when the nucleus is within the bud. Cdc42p, Cdc24p and the Cdc42p effector Cla4p are responsible for the phosphorylation-dependent bud localization of Lte1p (125), indicating that bud-tip activation of Cdc42p has an important role in MEN.

The G₂/M morphogenesis checkpoint is activated when components of the actin cytoskeleton are improperly assembled. Activation of the morphogenesis checkpoint occurs if the Swe1p kinase (Wee1 homolog) is stabilized. Swe1p, an integrator of upstream cell cycle events, is phosphorylated by the PAK Cla4p, the Polo kinase Cdc5p, and Clb2p-Cdc28p at the bud-neck, which results in its ubiquitination, proteasome-dependent degradation, and subsequent initiation of mitosis [reviewed in (168)].

The septin ring established in G₁ transforms into a collar at bud emergence and then splits into two rings that define mother, bud, and neck after chromosome and organelle segregation (97). Compromising septin integrity

results in depolarization of actin cables and a loss of the bud-specific localization of components of the exocyst, polarisome, actin patches (20) and components of the morphogenesis checkpoint [reviewed in (174)]. The septin collar filaments are parallel to the mother-bud axis and rotate 90° when the two rings are formed during cytokinesis (308). These results suggest that one function of septins at the mother-bud neck is to act as a diffusion barrier against asymmetrically transported, bud-specific proteins from re-entering the mother (see **Diffusion Restriction**).

The chitin synthase Chs2p is localized within the septin rings [reviewed in (43)], where it deposits chitin to form the primary septum, followed by contraction of the actomyosin ring to separate the plasma membranes of mother and bud (abscission). The NoCut pathway is a cell cycle checkpoint that determines if the chromosomes have cleared the midzone before initiating abscission. The Aurora kinase Ipl1p and the anillin-related Boi1p and Boi2p activate the checkpoint through a currently unknown mechanism, which interestingly does not appear to involve Ipl1p-dependent phosphorylation of Boi1p or Boi2p (216). Mother and bud then both form a secondary septum and removal of the primary septum through bud-specific enzyme secretion results in the separation the two cells [reviewed in (43)].

Analysis of the *cd42-V44A* allele indicated that Cdc42p has a role in the apical-isotropic switch that is likely exerted through its PAK effector Cla4p (248) and its role in triggering the morphogenesis checkpoint. *sec14^{ts}* (PI transfer

protein) mutants exhibit a delay in cytokinesis that is dependent on Cdc42p, Cla4p and Ste20p that can be suppressed by overexpression of Stt4p and Mss4p (129) [see **The Plasma Membrane Phosphoinositide Signaling Hub**]. This indicates that PE translocation back to the internal leaflet of the plasma membrane could signal Cdc42p removal from the bud tip (263) and PI(4,5)P₂ production at the mother-bud neck could be involved in its retargeting to the neck to facilitate passage through G₂/M. Bud tip maintenance of Cdc24p, and its phosphorylation by the PAK Cla4p, is dependent on interaction with Bem1p (42). Progressive phosphorylation of Cdc24p results in a decreased affinity for Bem1p and therefore has been proposed as a mechanism of Cdc24p removal from membranes (42) [see **Chapter 3**]. The mechanism by which Cdc24p is then re-targeted to the mother-bud neck is unknown.

Dissertation Research Summary

Cdc42p localizes around the entire plasma membrane (247), but is only activated at sites of polarized growth. Therefore, to ensure that Cdc42p is inactive (GDP-bound) around the cell periphery, but active (GTP-bound) at sites of polarized growth, Rdi1p and Cdc24p must be specifically targeted to these sites. Research on the role of Cdc42p in budded growth in yeast has primarily focused on alleles of *cdc42* that affect nucleotide or effector protein binding, and little was known about the role that regulators of Cdc42p have on its localization and activity, until recently. ***This dissertation research contributes to the understanding of how Cdc42p regulators affect its localization and activity by studying the spatial and temporal localization of the Cdc42p-Rdi1p complex and by identifying protein(s) involved in specifically targeting Cdc24p to sites of polarized growth.***

Cdc42p and Rdi1p were determined to interact diffusely throughout the cytoplasm of cells at all stages of the cell cycle and their interaction was enhanced at incipient bud sites, the tips and sides of small- and large-budded cells and at the mother-bud neck region. These results are consistent with the proposed functions of Rdi1p discussed within this dissertation: extraction of Cdc42p from the plasma membrane at sites of polarized growth, sequestering inactive Cdc42p in the cytoplasm and re-delivery of Cdc42p to sites of polarized growth.

Localization of Cdc24-647-854p to sites of polarized growth was assayed in strains that had gene deletions of proteins that were previously identified to interact or complex with Cdc24p. Aberrant, or no, localization of Cdc24-647-854p in these strains indicates that the missing protein has a role in targeting or maintenance of Cdc24p at sites of polarized growth. Boi2p, Ent2p, Hua1p and Rsc2p were found to have a role in the targeting and/or maintenance of Cdc24p at sites of polarized growth.

Potential CDK phosphorylation sites within the targeting and anchoring domain were mutated to a non-phosphorylatable residues. Cdc24-647-854^{S697A}p and Cdc24-647-854^{S697A, T704p}p localized abnormally to the mother-bud neck of pre-anaphase cells. The same abnormal localization of the targeting and anchoring domain was observed in *tos2Δ skg6Δ* mutants. Taken together, these results suggest that phosphorylation of this domain, and its ability to interact with Tos2p and Skg6p at sites of polarized growth, are required for maintenance at bud tips.

CHAPTER 2

USE OF BIMOLECULAR FLUORESCENCE COMPLEMENTATION TO STUDY INTERACTIONS BETWEEN SACCHAROMYCES CEREVISIAE CDC42P AND RDI1P

My contributions to this chapter include experimental design, execution and writing.

Use of bimolecular fluorescence complementation to study *in vivo* interactions between *Saccharomyces cerevisiae* Cdc42p and Rdi1p

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Summary

S. cerevisiae Cdc42p functions as a GTPase molecular switch, activating multiple signaling pathways required to regulate cell cycle progression and the actin cytoskeleton. Regulatory proteins control its GTP-binding and hydrolysis and its subcellular localization, ensuring that Cdc42p is appropriately activated and localized at sites of polarized growth during the cell cycle. One of these, the Rdi1p guanine nucleotide dissociation inhibitor (GDI), negatively regulates Cdc42p by extracting it from cellular membranes. In this study, the technique of bimolecular fluorescence complementation (BiFC) was used to study the dynamic *in vivo* interactions between Cdc42p and Rdi1p. The BiFC data indicated that Cdc42p and Rdi1p interacted in the cytoplasm and around the periphery of the cell at the plasma membrane, and that this interaction was enhanced at sites of polarized cell growth during the cell cycle, *i.e.*, incipient bud sites, tips and sides of small- and medium-sized buds, and the mother-bud neck region. In addition, a ring-like structure containing the Cdc42p-Rdi1p complex transiently appeared following release from a G₁-phase cell cycle arrest. A homology model of the Cdc42p-Rdi1p complex was used to introduce mutations that were predicted to affect complex formation. These mutations resulted in altered BiFC interactions, restricting the complex exclusively to either the plasma membrane or the cytoplasm. Data from these studies have facilitated the temporal and spatial modeling of Rdi1p-dependent extraction of Cdc42p from the plasma membrane during the cell cycle.

Introduction

Highly conserved among eukaryotes, the Rho GTPase Cdc42 functions as a binary molecular switch capable of activating signaling pathways that regulate polarized cell growth, cell-cycle progression, gene transcription, and vesicle trafficking [reviewed in (78, 79, 145)]. Rho GTPases cycle between active (GTP-bound) and inactive (GDP-bound) states in a spatially and temporally controlled manner, which is achieved through interactions with three classes of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (30).

Three mammalian RhoGDIs have been identified (169, 301, 324); RhoGDI α is ubiquitously expressed, while RhoGDI β and RhoGDI γ exhibit tissue-specific expression. *S. cerevisiae* has one RhoGDI, Rdi1p, which has 36% amino-acid identity with human RhoGDI α (188). RhoGDIs have multiple functions within cells. They antagonize the action of GEFs by inhibiting GDP dissociation (301), and they antagonize GAPs by inhibiting GTP hydrolysis (59). In addition, RhoGDIs extract Rho GTPases from membranes (172), sequestering them in the cytoplasm (219). RhoGDIs contain two functional domains: an amino-terminal “regulatory arm” that interacts with the Switch I and Switch II domains of the Rho GTPase, and a carboxyl-terminal “geranylgeranyl (GG)-binding domain” that interacts with the geranylgeranyl moiety bound to the carboxyl terminus of the Rho GTPase (124). Interactions between the

geranylgeranyl moiety and the RhoGDI GG-binding domain are believed to facilitate the extraction of Rho GTPases from cellular membranes (124, 235).

In vitro kinetic studies utilizing fluorescence resonance energy transfer (FRET) between human Cdc42 and RhoGDI suggested a two-step model for RhoGDI extraction of Cdc42 from membranes (215). This model was supported by the crystallographic structure of the human GDP-bound Cdc42-bovine RhoGDI complex (124). In the first step of this model, the carboxyl-terminal GG-binding domain of RhoGDI interacts with Cdc42. This interaction is also believed to guide the RhoGDI amino-terminal regulatory arm into contact with the Cdc42 Switch I and II regions, thus preventing Cdc42 from interacting with GEFs, GAPs and downstream effector proteins. In the second step, the Cdc42 geranylgeranyl moiety isomerizes from the membrane into the RhoGDI GG-binding domain, resulting in extraction from the membrane and shielding of the geranylgeranyl moiety from the aqueous environment (219). Extraction is facilitated by the interaction of the Cdc42 carboxyl-terminal polylysine region (Cdc42 K183-187) with an acidic patch in the GG-binding domain of the RhoGDI.

Current models (72, 219) suggest that the GDP-bound GTPase is complexed with the RhoGDI in the cytoplasm. A signal triggers the translocation of the complex to a cellular membrane, where a displacement factor facilitates GTPase release from the RhoGDI. The exchange of GDP for GTP is then catalyzed by GEFs and the active, GTP-bound GTPase interacts with downstream effector proteins to activate various signaling pathways. Upon

signal down-regulation, RhoGDIs extract either the GTP-bound GTPase or, after GAP-mediated GTP hydrolysis, the GDP-bound GTPase from the membrane. The cytoplasmic RhoGDI-GTPase complex may be a source of readily activatable GTPase or it may be shuttled to other membranes within the cell (176). The RhoGDI-Rho GTPase complex can also be directly involved in signaling; for example, the RhoGDI-Rac complex activates NADPH oxidase (71) and the RhoGDI β -Cdc42 complex activates phospholipase C- β_2 (134).

S. cerevisiae Rdi1p is a non-essential protein that, when over-expressed, causes growth inhibition, presumably by extracting Cdc42p from membranes and sequestering it in the cytoplasm (188, 249). Rdi1p has been shown to co-immunoprecipitate with Cdc42p from cytoplasmic fractions (155). In addition, a GFP-tagged Rdi1p was previously shown to be a cytoplasmic protein that only consistently localized to the plasma membrane at the tips of small-sized buds and at the mother-bud neck region (249). Cdc42p was found in both soluble and particulate fractions (333), suggesting that it localized to both the cytoplasm and membranes. In addition, GFP-Cdc42p was previously shown to localize around the plasma and internal membranes and was observed to cluster at incipient bud sites, tips and sides of small- and medium-sized buds, and at the mother-bud neck region (247). Rdi1p and Cdc42p, therefore, co-localized in the cytoplasm, at the tips and sides of enlarging buds, and at the mother-bud neck region.

The new technique of bimolecular fluorescence complementation (BiFC) was utilized to determine if Rdi1p and Cdc42p actually interacted at sites where

they co-localized, and to study the dynamic localization of the Cdc42p-Rdi1p complex during the cell cycle. BiFC enables visualization of protein-protein interactions *in vivo* (130, 151). In this approach, the two non-fluorescent halves of the GFP-variant yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) are fused separately to two potential interacting proteins. Interaction of the two fusion proteins leads to reconstitution of the GFP variant, and hence fluorescence. BiFC has recently been used to successfully dissect interactions between cytokine receptors (93), cytochromes (224), transcription factors (122), and proteins involved in *S. cerevisiae* cytokinesis (25).

In this study, BiFC data indicated that Cdc42p and Rdi1p interacted in the cytoplasm and around the periphery of the cell at the plasma membrane. In addition, there were enhanced interactions at incipient bud sites, the tips and sides of small- and medium-sized buds, and at the mother-bud neck region. These interactions were confirmed using time-lapse photomicroscopy and cell-cycle synchrony, which also revealed the presence of a novel Cdc42p-Rdi1p complex in a transient ring-like structure following release from G₁ arrest. Genetic analyses indicated that mutations predicted to affect Rdi1p regulatory arm function [*rdi1(D38A)*, *rdi1(W44A)*, and *cdc42(R66E)*] restricted Cdc42p-Rdi1p BiFC interactions exclusively to the plasma membrane, as did the *rdi1(P167K)* mutation, which was predicted to interfere with the binding of the Cdc42p geranylgeranyl moiety. The *cdc42(K183-187Q)* and *cdc42(C188S)* mutations, which affect the localization of Cdc42p to the plasma membrane,

restricted Cdc42p-Rdi1p interactions to the cytoplasm. These data have provided valuable insight into the mechanisms by which Rdi1p interacts with Cdc42p during the cell cycle, and have highlighted the overall usefulness of BiFC as a technique to study *in vivo* protein-protein interactions.

Materials and Methods

DNA manipulations

p415MET(YN-*RDI1*) and p416MET(CC-*CDC42*) were made by D-loop PCR utilizing primers (Invitrogen, Carlsbad, CA) that removed aa 155-238 from YFP in p415MET(YFP-*RDI1*) (249) and aa 1-154 from CFP in p416MET(CFP-*CDC42*) (247). The 8-Ala linker remained intact at the carboxyl terminus of both constructs. p416MET(CC-*CDC42*) was converted to p416MET(YC-*CDC42*) by site-directed mutagenesis, changing Thr203 of CFP to Tyr. Subcellular localization of fluorescent protein-tagged wild-type and mutant proteins was performed with transformants containing either p415MET(GFP-Rdi1p) or p416MET(GFP-Cdc42p) except for p416MET(CFP-Cdc42-R66Ep). Primer sequences and cycling conditions are available upon request. Site-directed mutagenesis was performed in these plasmids and in pKT10(*RDI1*) (188) utilizing primers (Invitrogen) with the QuikChange Kit (Stratagene, La Jolla, CA) following manufacturer's instructions, with the exception of the creation of p416MET(CC-*cdc42-C188S*). This primer set (SigmaGenosys, St. Louis, MO) required an annealing temperature of 52°C. All mutations were verified by the Vermont Cancer Center DNA Sequencing Facility. p414MET(YN-*RDI1*) was made by releasing YN-*RDI1* from p415MET(YN-*RDI1*) with *Spe1* and *Xho1* and inserting into p414MET cut with *Spe1* and *Xho1*.

Reagents, media and strains

Growth media, maintenance of bacterial and yeast strains, and yeast transformations were described by Sambrook *et al.* (264) and Sherman *et al.* (274). Low fluorescence (LF) yeast nitrogen base, as described by Sheff and Thorn (272), was used in all media for microscopy. The yeast strains used for microscopy, immunoblotting, and cell synchrony experiments were BJ5459 (*MATa pep4::HIS3 prb1-Δ1.6R his3Δ200 lys2-801 trp1 ura3-52 can1 leu2Δ1*) (146) and 1607-5D (*MATα bar1 cln1 cln2 cln3 ura3 his2 ade1 arg4 trp1 leu2::LEU2::GAL1::CLN3*) (63).

Fluorescence Microscopy

BJ5459 cells transformed with BiFC or GFP-variant (for subcellular localization studies) plasmids were grown in the appropriate LF synthetic complete (SC) liquid media to mid-log phase, collected by centrifugation, sonicated, and viewed using Differential Interference Contrast optics. Fluorescence microscopy on an E400 Nikon microscope (Omega Optical, Brattleboro, VT) utilized an Omega XF100 optical filter cube for GFP-tagged proteins, a Chroma Cyan GFP V2 (31044 V2) optical filter cube for CFP-tagged proteins, and a Chroma Yellow GFP (41028) optical filter cube for both YFP-tagged proteins and BiFC constructs. Exposure times for BiFC constructs were empirically determined: YN/CC partners – 25 sec.; YN/YC partners – 8 sec.

Immunoblot analysis

Total protein was isolated from BJ5459 or 1607-5D cells expressing the BiFC constructs previously described. A 1:5000 dilution of polyclonal rabbit anti-GFP α -Av antibody (BD Biosciences, San Jose, CA) and a 1:8000 dilution of goat α -rabbit horseradish peroxidase (Sigma, St. Louis, MO) were used to detect YN-, CC- and YC-fusion proteins as previously described (293).

Homology modeling of Cdc42p and Rdi1p

The SWISS-MODEL (<http://swissmodel.expasy.org/workspace/>) first approach mode was utilized to build a homology model (105, 231, 270) of the Cdc42p-Rdi1p complex from the template structure (PDB accession code 1DOA) of the human GDP-bound Cdc42-bovine RhoGDI complex (124). The first three amino-terminal amino acids of Rdi1p were added to the returned structure using the build function of SwissPDB Viewer.

Results

A Cdc42p-Rdi1p BiFC signal was observed in the cytoplasm and around the periphery of the cell at the plasma membrane.

BiFC was used to determine if Cdc42p and Rdi1p physically interacted at sites where they have been shown to co-localize: in the cytoplasm, at the tips of small-sized buds, and at the mother-bud neck region (247, 249). The BiFC constructs were generated by fusing Rdi1p to the amino terminus of YFP (aa 1-154; YN-Rdi1p) and fusing Cdc42p to the carboxyl terminus of either CFP or YFP (aa 155-238; CC-Cdc42p or YC-Cdc42p). A Cdc42p-Rdi1p BiFC signal was observed in the cytoplasm and around the periphery of the cell at the plasma membrane (Fig. 10A). An enhanced BiFC signal was observed at incipient bud sites, the tips and sides of small- and medium-sized buds, and at the mother-bud neck region (arrows, Fig. 10A). The same BiFC signal pattern was observed with YN-Rdi1p and both CC-Cdc42p and YC-Cdc42p (Fig. 10A).

To confirm that the observed BiFC signals depended solely on Cdc42p-Rdi1p interactions, wild-type and mutant YN-Rdi1p, YC-Cdc42p, and CC-Cdc42p were expressed in BJ5459 cells. Cells expressing either wild-type YN-Rdi1p, CC-Cdc42p, or YC-Cdc42p alone did not exhibit fluorescence above the background of BJ5459 cells (Fig. 10B), verifying that the YN, YC, and CC fluorophore fragments fused to Rdi1p and Cdc42p were non-fluorescent. In addition, a BiFC signal was not observed when YN-Rdi1p was expressed with

either CC-Cdc42-D118Ap or YC-Cdc42-T17Np, two *cdc42* mutants that do not bind guanine nucleotide and, therefore, do not interact with RhoGDI (92, 332) (Fig. 10C). However, a BiFC signal with a wild-type localization pattern was observed when YN-Rdi1p was expressed with CC-Cdc42-F28Lp, a 'fast-cycling' *cdc42* mutant that can spontaneously exchange guanine nucleotide (177) and bind to RhoGDI (176) (Fig. 10C). The observation that single point mutations in Cdc42p known to abolish interaction with RhoGDI resulted in a lack of a BiFC signal indicated that specific interactions between Cdc42p and Rdi1p, and not non-specific interactions between the YN and CC or YC fluorophore fragments, were responsible for the BiFC signals.

The absence of a BiFC signal could also be the result of instability of the fusion proteins. Immunoblot analysis indicated that in cells that did not display a BiFC signal, YN-Rdi1p, CC-Cdc42p and YC-Cdc42p were stably expressed at levels comparable to that observed in cells where they were co-expressed and exhibited a BiFC signal (Fig. 10D). CC-Cdc42-F28Lp and CC-Cdc42-D118Ap were also stably expressed at levels comparable to wild-type fusion proteins (Fig. 10D). Therefore, absence of a BiFC signal was not the result of instability of any of these proteins.

A BiFC signal was observed in a transient ring-like structure in G₁ phase.

Time-lapse photomicroscopy together with cell-cycle synchrony was utilized to follow Cdc42p-Rdi1p interactions through the cell cycle. 1607-5D cells containing YN-Rdi1p and YC-Cdc42p were arrested in G₁ phase by glucose-induced depletion of the G₁ cyclin Cln3p in a *cln1Δ cln2Δ* background (63). Following release from G₁ arrest, cells were followed for ~280 min. in either liquid media (data not shown) or on agar slides (n=25; Fig. 11A). As seen with the asynchronous population of cells, a Cdc42p-Rdi1p BiFC signal was observed in the cytoplasm and around the periphery of the cell at the plasma membrane throughout the cell cycle. In cells grown in liquid culture, an enhanced BiFC signal was observed at incipient bud sites ~5-30 min. following release (data not shown), whereas the appearance at incipient bud sites was slightly delayed until ~60-110 min. following release on agar slides (Fig. 11A; arrows, 105 min.). Enhanced BiFC signals were also observed in small- and medium-sized buds, and at the mother-bud neck region (Fig. 11A; arrows, 245 min.). These results are consistent with the notion that an increase in Rdi1p at sites of polarized growth is required to remove clustered Cdc42p from membranes when polarized cell growth is no longer required (249).

A BiFC signal was also observed in a ring-like structure following release from G₁ arrest (Fig. 11B, galactose). Approximately 50% of cells (n = 100) released into galactose-containing liquid media had this ring-like structure

present within ~5 min. of release. Cells ($n = 100$) that were arrested in G₁ phase by growth in glucose media did not display this ring-like structure. This structure was transient, in that it disappeared in all cells examined within ~5 min. (data not shown). This observation was interesting in that BiFC complexes are irreversible *in vitro* (see Discussion). However, these data suggest that the Cdc42p-Rdi1p BiFC complex was reversible and dynamic in nature.

Homology modeling of the Cdc42p-Rdi1p complex facilitated a genetic analysis of Cdc42p-Rdi1p BiFC interactions.

To facilitate the genetic analysis of Cdc42p-Rdi1p complex formation, an homology model of the *S. cerevisiae* Cdc42p-Rdi1p complex (Fig. 12) was generated based on the crystal structure of the human Cdc42-bovine GDI complex (124). Human and *S. cerevisiae* Cdc42p share 80.7% amino-acid sequence identity, whereas the bovine GDI and Rdi1p are 38.6% identical. Automated homology modeling is believed to be reliable when the target and template have greater than 50% amino-acid identity (11). Although the bovine GDI-Rdi1p identity falls below this threshold, the homology model generated was sufficient for guiding site-directed mutagenesis studies, as the critical residues at the interface of human Cdc42p and RhoGDI were conserved. Therefore, mutations predicted to affect the functional interaction between the proteins were made in the Rdi1p regulatory arm (D38A, S40A, W44A and Δ 37-47) and GG-

binding domain (P167K), and in the Cdc42p Switch II domain (R66E) and geranylgeranylation site (C188S, K183-187Q) (Fig. 12; Table 9).

Mutations in the Rdi1p regulatory arm and GG-binding domain and the Cdc42p Switch II domain did not block Cdc42p-Rdi1p complex formation.

Previous studies showed that plasma membrane localization of GFP-Cdc42p depended on both geranylgeranylation of a carboxyl-terminal Cys residue and the polylysine region (KKSKK¹⁸⁷) next to the Cys residue (66, 247). It is believed that a carboxyl terminal “acidic patch” within the RhoGDI GG-binding domain can compete with negatively charged membrane phospholipids for binding to the positively charged polylysine region, thereby facilitating GDI-dependent extraction of Cdc42p from membranes (124). Changing the four Lys residues to Gln (K183-187Q) resulted in a BiFC signal between YN-Rdi1p and CC-Cdc42-K183-187Qp, indicating that altering the Lys residues did not block complex formation, but the BiFC signal was only observed in the cytoplasm (Fig. 13A). As GFP-Cdc42-K183-187Qp localized to the cytoplasm and internal membranes but not to the plasma membrane [(247); Table 9], this result suggested that Cdc42-K183-187Qp could interact with Rdi1p in the cytoplasm, but that the Cdc42-K183-187Qp-Rdi1p complex could not be targeted to the plasma membrane.

The proximity of the Cdc42 Switch II Arg66 residue with amino acids in both the RhoGDI regulatory arm and the external face of the GG-binding domain

(Fig. 12) suggested that this amino acid played an important role in stability of the Cdc42p-Rdi1p complex (72). A BiFC signal was observed between YN-Rdi1p and CC-Cdc42-R66Ep, indicating that the R66E mutation does not block complex formation, but in this case, the BiFC signal was predominantly observed at the plasma membrane (Fig. 13A). Since GFP-Cdc42-R66Ep localized normally to the cytoplasm and to plasma and internal membranes (Table 9), these results suggested that either Cdc42-R66Ep could only interact with Rdi1p at the plasma membrane and not in the cytoplasm, or that Rdi1p could not extract Cdc42-R66Ep from the plasma membrane.

To address these possibilities, the *cdc42*(R66E) (plasma membrane BiFC) and *cdc42*(K183-187Q) (cytoplasmic BiFC) mutations were combined. If the *cdc42*-R66E mutation prevented formation of a stable complex with Rdi1p in the cytoplasm, then the double mutation would result in a complete loss of interactions with Rdi1p. A BiFC signal with wild-type localization was only observed in 10% of cells that expressed YN-Rdi1p and CC-Cdc42-K183-187Q,R66Ep (Fig. 13A), suggesting that the R66E mutation does interfere with the formation of a Cdc42p-Rdi1p complex in the cytoplasm. However, this result does not rule out the possibility that Rdi1p cannot effectively extract Cdc42-R66Ep from the plasma membrane (see below).

Geranylgeranylation of the Rho GTPase Rac is essential for high-affinity binding to RhoGDI (175) and a geranylgeranylation-defective Cdc42-C188Sp does not localize to the plasma membrane (247, 333). Therefore, it was

predicted that CC-Cdc42-C188Sp and YN-Rdi1p would have little or no interactions, and, if observed, would be localized to the cytoplasm. In fact, an attenuated BiFC signal between Cdc42-C188Sp and Rdi1p was only observed in the cytoplasm (Fig. 13B), indicating that geranylgeranylation of Cdc42p was important but not essential for interactions with Rdi1p in the cytoplasm.

The Cdc42p-Rdi1p complex model was examined to determine if amino acid(s) at the opening of the Rdi1p carboxyl-terminal GG-binding domain could be mutated such that insertion of the geranylgeranyl moiety would be prevented. Mutating Pro167 to Lys was predicted to provide both steric and charge hindrance to insertion of the large 20-carbon, hydrophobic geranylgeranyl moiety (Fig. 13B). Interestingly, a BiFC signal between YN-Rdi1-P167Kp and YC-Cdc42p was observed (Fig. 13B), indicating that the Rdi1-P167Kp mutant protein could still interact with Cdc42p. However, the BiFC signal was only observed at the plasma membrane, suggesting that Rdi1-P167Kp could not extract Cdc42p from membranes, possibly because it could not bind the geranylgeranyl moiety (see below and Discussion). In addition, a predominantly cytoplasmic BiFC signal was observed between YN-Rdi1-P167Kp and YC-Cdc42-C188Sp (data not shown), indicating that Rdi1-P167Kp could bind to Cdc42p lacking a geranylgeranyl moiety.

The Asp45 and Ser47 residues in the bovine RhoGDI regulatory arm interact with the Thr35 and Val36 residues, respectively, in the Cdc42 Switch I domain (72, 124). Also, the Tyr51 regulatory arm residue interacts with

conserved Tyr64, Leu67, and Leu70 hydrophobic residues in Cdc42 (124). The equivalent residues in Rdi1p (Asp38, Ser40, and Trp44) were mutated to Ala residues. A BiFC signal was observed in cells expressing either YN-Rdi1-D38Ap, YN-Rdi1-W44Ap, or YN-Rdi1-S40Ap together with YC-Cdc42p (Fig. 13C), indicating that these mutations do not interfere with Cdc42p binding. A BiFC signal was also observed between YN-Rdi1-D38Ap and CC-Cdc42-R66Ep (data not shown). Although the Cdc42p-Rdi1-S40Ap BiFC signal showed a wild-type localization pattern, the Cdc42p-Rdi1-D38Ap, Cdc42p-Rdi1-W44Ap, and Cdc42-R66Ep-Rdi1-D38Ap BiFC signals were only observed at the plasma membrane (Fig. 13C and data not shown), suggesting that these mutant Rdi1p may not be able to extract Cdc42p from membranes (see below).

Deletion of the amino-terminal 59 amino acids of bovine RhoGDI, which includes the regulatory arm, did not interfere with binding to Cdc42 but did block membrane extraction of Cdc42 (101). Therefore, regulatory arm amino acids 37-47 of Rdi1p, which encompass the α C helix that lies in proximity to the SwitchI/II domain of Cdc42p, were deleted to assess their function in complex formation. Interestingly, a BiFC signal between YN-Rdi1- Δ 37-47p and YC-Cdc42p was observed (Fig. 13C), again indicating that deletion of the Rdi1p regulatory arm did not interfere with Cdc42p binding, but the BiFC signal was only observed at the plasma membrane, suggesting that the mutant Rdi1- Δ 37-47p could not extract Cdc42p from the membrane.

GFP-Cdc42p release from membranes can be stimulated by expression of Rdi1p regulatory arm mutants.

Overexpression of wild-type Rdi1p resulted in two cellular phenotypes: inhibition of cell growth and loss of GFP-Cdc42p membrane localization (155, 188, 249). Therefore, these two phenotypes were used as assays to examine whether the effects of mutations that restricted Cdc42p-Rdi1p BiFC interactions to the plasma membrane [*cdc42*(R66E), *rdi1*(W44A), and *rdi1*(P167K)] were due to an inability of Rdi1p to extract Cdc42p from membranes.

In the growth-inhibition assay, cells expressing wild-type Rdi1p or mutants Rdi1-W44Ap, Rdi1-P167Kp, or Rdi1-S40Ap (a control that showed wild-type BiFC signal patterns) under the control of a galactose-inducible promoter could grow on repression media (glucose), but could not grow on derepression media (galactose) (Fig. 14A). This result indicated that the three *rdi1* mutants had retained the ability to cause growth arrest. However, in the membrane-extraction assay, cells expressing Rdi1-S40Ap showed predominately cytosolic GFP-Cdc42p localization, whereas cells expressing Rdi1-W44Ap or Rdi1-P167Kp displayed predominantly plasma membrane GFP-Cdc42p localization (Fig. 14B, right panels). Taken together, these data suggested that Rdi1-S40Ap had retained the ability to extract Cdc42p from membranes, thereby causing growth arrest, but Rdi1-W44Ap and Rdi1-P167Kp had lost that ability. Therefore, the nature of the growth arrest seen with Rdi1-W44Ap and Rdi1-P167Kp was unclear, but may be due to the generation of dominant-negative complexes at the

membrane as Cdc42p-Rdi1-W44Ap and Cdc42p-Rdi1-P167Kp BiFC signals were restricted to the plasma membrane.

In the membrane-extraction assay, expression of Rdi1p resulted in predominantly cytosolic localization of wild-type GFP-Cdc42p, but predominantly plasma membrane localization of GFP-Cdc42-R66Ep (Fig. 14B). This result suggested that Rdi1p could not extract mutant Cdc42-R66Ep from the plasma membrane, possibly generating a dominant-negative complex at the membrane, as cells expressing wild-type Rdi1p with mutant Cdc42-R66Ep could not grow on derepression media (galactose; Fig. 14A). These data, taken together with the BiFC data, indicated that the restriction of the Cdc42-R66Ep-Rdi1p BiFC signal to the plasma membrane was most likely due to the inability of Rdi1p to extract Cdc42-R66Ep from membranes.

Discussion

During the *S. cerevisiae* cell cycle, Cdc42p is required for actin rearrangements that occur at sites of polarized cell growth. Consistent with this cell cycle role, functional GFP-Cdc42p localized around the periphery of the cell at the plasma membrane and was observed to cluster at polarized growth sites, including incipient bud sites, tips and sides of small- and medium-sized buds, and the mother-bud neck region (247). Cdc42p was also observed in the cytoplasm (333), and, hence, a dynamic equilibrium must exist between cytoplasmic and membrane-bound pools. However, the mechanisms by which Cdc42p is trafficked from the cytoplasm to the plasma membrane and sites of polarized cell growth, and subsequently extracted from membranes to cytoplasmic pools later in the cell cycle, have remained elusive (247).

It is clear that mammalian RhoGDI proteins play an important role in the trafficking and extraction of Rho GTPase from membranes. As *S. cerevisiae rdi1Δ* cells are viable (188, 249) and GFP-Cdc42p localized and clustered normally in *rdi1Δ* cells (T. Richman and D. I. Johnson, unpublished results), the ability of Rdi1p to traffic and/or remove Cdc42p from the plasma membrane is not essential for cell growth. Although Cdc42p could localize to the plasma membrane independent of Rdi1p, Rdi1p was able to extract GFP-Cdc42p from the plasma membrane (249). Consistent with this function, GFP-tagged Rdi1p localized to the plasma membrane at the tips of small-sized buds and at the mother-bud neck region, as well as to the cytoplasm (249).

The observed Cdc42p-Rdi1p BiFC interaction patterns described herein were consistent with the localization of the individual GFP-tagged proteins with three notable exceptions: (i) BiFC interactions were evident between Cdc42p and Rdi1p around the entire periphery of the cell at the plasma membrane instead of just at sites of polarized growth; (ii) enhanced BiFC signals were observed at incipient bud sites where GFP-Rdi1p did not consistently localize (249); and (iii) the proteins interacted around the tips and sides of large-sized buds (arrows; Fig. 11A). There are several possible explanations for these differences that will be explored in future studies: (a) the BiFC technique was able to capture a rapid transient event in which the proteins interacted around the entire periphery of the cell and at incipient bud sites; (b) the reconstituted fluorophore irreversibly conjoined the fusion proteins, and they diffused away from sites of polarized growth, but could not be extracted from membranes in large-sized buds; and/or (c) expression of the proteins overwhelmed a potential Rdi1p displacement factor, thereby causing the complex to remain membrane-associated. Although it has been proposed that cytoplasmic RhoGDI shuttles Rho GTPases to other internal membranes within the cell (72), a Cdc42p-Rdi1p BiFC signal was not observed at internal membranes, even though GFP-Cdc42p has been localized to these membranes. The cytoplasmic Cdc42p-Rdi1p complex, therefore, may not be shuttling Cdc42p to internal membranes but just serving as a source of readily activatable GTPase (219). Time-lapse photomicroscopy of G₁-synchronized cells indicated that Cdc42p interacted with

Rdi1p in the cytoplasm and around the periphery of the cell during a G₁ arrest and subsequently throughout the cell cycle, indicating that a Cdc42p-Rdi1p complex does not need to be specifically targeted to the plasma membrane following START. Surprisingly, within 5 minutes of release from the cyclin-depletion-induced cell cycle arrest, a Cdc42p-Rdi1p complex appeared in a ring-like structure, and this complex disappeared within ~5 minutes. The rapid disappearance of the ring-like BiFC structure was presumably due to the disassembly of the Cdc42p-Rdi1p complex. Whereas the reassembly of GFP from two nonfluorescent halves is fundamentally irreversible *in vitro*, with the $t_{1/2}$ of dissociation of the halves being ~10 years without a denaturing agent (184), disassembly of other BiFC complexes has been previously observed *in vivo* (68, 109).

There are several mechanisms that may account for the dissociation of the Cdc42p-Rdi1p ring-like structure following release from G₁ arrest: phosphorylation of the RhoGDI, RhoGDI displacement factors, and/or phospholipid-induced dissociation (72). Mammalian PAK (*p*21-activated kinase) phosphorylated RhoGDI α on two Ser residues, which decreased the affinity of RhoGDI α for Rac1 (70). RhoGDI protein displacement factors are members of the ERM (ezrin, radixin, moesin) superfamily, which link actin filaments to plasma membrane proteins (38, 74, 267). Although ERM domain-containing proteins have not been identified in *S. cerevisiae* (38), it is likely that additional proteins mediate interactions between Cdc42p and Rdi1p. This supposition is supported

by the observation that deletion of the Cdc42p Rho-insert domain, an 13 amino-acid domain (aa 122-134) not directly involved in protein-protein interactions with Rdi1p (see Fig. 12), prevented Rdi1p-dependent membrane extraction of Cdc42p (249). Phospholipids have been shown to disrupt a cytosolic Rac-RhoGDI complex (58) and have been linked to increasing CD44's affinity for ERM (121), presumably resulting in loss of Rac inhibition and delivery of Rho GTPases to membrane signaling complexes. Studies are currently underway in the laboratory to address these three possibilities.

Cdc42-RhoGDI binding has been proposed to occur in two steps (73). Initially, amino acids in the RhoGDI regulatory arm interact with amino acids in the Cdc42 Switch I/II domains, thereby detecting the nucleotide status of the GTPase. This is followed by an interaction between amino acids on the external face of the GG-binding domain and amino acids in the Switch II domain, which positions the complex at the membrane for extraction. Surprisingly, neither mutations in (D38A, S40A, W44A), or deletion of (Δ 37-47), the Rdi1p regulatory arm, nor mutations in the GG-binding domain (P167K) alone could block the formation of a Cdc42p-Rdi1p complex (Fig. 13; Table 9). These results indicated that neither domain was necessary for binding Cdc42p and that both domains most likely act cooperatively to form a Cdc42p-Rdi1p complex. However, the W44A mutation did affect the ability of Rdi1p to extract Cdc42p from the plasma membrane, suggesting that regulatory arm interactions play a role in positioning Rdi1p in an extraction-competent conformation.

Mutations predicted to affect geranylgeranyl binding resulted in a shift of the Cdc42p-Rdi1p complex to either the cytoplasm [*cdc42*(K183-187Q) and *cdc42*(C188S)] or the plasma membrane [*rdi1*(P167K)]. The persistence of the Cdc42-K183-187Qp-Rdi1p and Cdc42-C188Sp-Rdi1p complexes in the cytoplasm was most likely due to the inability of these mutant Cdc42p to target to the membrane (66, 247). The inability of Rdi1-P167Kp to extract GFP-Cdc42p from the plasma membrane (Fig. 14) is the most likely explanation for the persistence of the Cdc42p-Rdi1-P167Kp complex at the plasma membrane. Previous studies showed that the RhoGDI-Ile177 residue (located at the base of the GG-binding pocket) was required for high-affinity geranylgeranyl binding and RhoGDI-dependent extraction of Cdc42 from membranes (124, 235). Mutation of this residue to Asp resulted in a dominant-negative protein that formed a complex with Cdc42 at the plasma membrane (287). It is likely that the Cdc42p-Rdi1-P167Kp complex is analogous, since Rdi1-P167Kp should not be able to bind the geranylgeranyl moiety and it formed a dominant-negative Cdc42p complex restricted to the plasma membrane.

BiFC data indicated that the mutant Cdc42-R66Ep could interact with Rdi1p, in contrast to previous studies in mammalian cells (91, 176), but the membrane-extraction and growth inhibition assays indicated that Rdi1p could not extract Cdc42-R66Ep from the plasma membrane (Fig. 14), which agrees with analysis of the mammalian Cdc42-R66E mutant protein. This inability to be extracted from membranes may be due to the role of the Cdc42-R66 residue in

binding to the external face of the Rdi1p GG-binding domain, leading to the proper positioning of the Cdc42p-Rdi1p complex at the membrane (73).

Taken together, these data suggest a model for dynamic Cdc42p-Rdi1p interactions throughout the cell cycle. Rdi1p is likely to play an important, although not essential, role in trafficking Cdc42p to and from the plasma membrane at sites of polarized cell growth during the cell cycle. Rdi1p likely functions in the extraction of Cdc42p from membranes at these sites upon the completion of polarized cell growth following the apical-isotropic switch in S/G₂ phase. Rdi1p does not seem to restrict Cdc42p to polarized growth sites in *S. cerevisiae*, in contrast to other organisms such as *Arabidopsis*, in which a RhoGDI is required to spatially restrict ROP GTPases to sites of polarized growth (46). These results also suggested that the Rdi1p amino-terminal regulatory arm and the carboxy-terminal GG-binding domain act cooperatively to bind and extract Cdc42p from membranes, an observation that may shed new light on the proposed two-step model for Rho GTPase-RhoGDI binding. Additional BiFC and genetic analyses should provide important new insights into the mechanisms by which RhoGDIs bind and extract Rho GTPases from cellular membranes.

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Table 9: Summary of BiFC interactions.

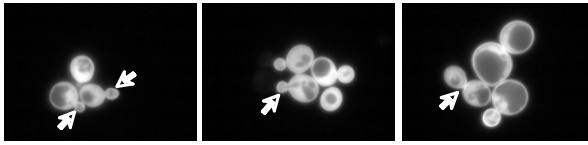
	Interface/function affected	Localization ^a	BiFC Signal ^a
Cdc42p	wild-type	CPI ^b	CP
Cdc42 ^{D118A} p	nucleotide binding	CPI	—
Cdc42 ^{T17N} p	nucleotide binding	CPI	—
Cdc42 ^{F28L} p	nucleotide binding	CPI	CP
Cdc42 ^{R66E} p	GG-binding domain; Switch II	CPI	P
Cdc42 ^{K183-187Q} p	acidic patch/polylysine	C	C
Cdc42 ^{K183-187Q, R66E} p	see above	nt	— (90%) CP (10%)
Cdc42 ^{C188S} p	geranylgeranylation	C ^b	C
Rdi1p	wild-type	CP ^{C*}	CP
Rdi1 ^{D38A} p	regulatory Arm/Switch I	nt	P
Rdi1 ^{S40A} p	regulatory Arm/Switch I	nt	CP
Rdi1 ^{W44A} p	regulatory Arm/Switch I	CP	P
Rdi1 ^{Δ37-47} p	regulatory Arm/Switch I, II	CP	P
Rdi1 ^{P167K} p	GG-binding domain	CP	P
Cdc42 ^{C188S} p + Rdi1 ^{P167K} p	see above	C CP	C

Unless otherwise stated, all Cdc42p mutations were tested for BiFC interactions with wild-type Rdi1p, and all Rdi1p mutations were tested for BiFC interactions with wild-type Cdc42p.

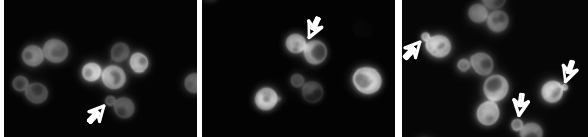
Figure 10. BiFC interactions between Cdc42p and Rdi1p. **(A)** BJ5459 cells expressing YN-Rdi1p and either CC-Cdc42p or YC-Cdc42p were grown in low fluorescent SC media –Met to mid-log phase and observed by fluorescence microscopy. Arrows indicate enhanced BiFC signal at the tips and sides of small- and medium-sized buds, and at the mother-bud neck region. **(B)** BJ5459 cells expressing either YN-Rdi1p, CC-Cdc42p, or YC-Cdc42p alone were grown and observed as in **(A)**. **(C)** BJ5459 cells expressing YN-Rdi1p and either CC-Cdc42-F28Lp, CC-Cdc42-D118Ap, or CC-Cdc42-T17Np were observed as in **(A)**. **(D)** Immunoblot analysis of Cdc42p and Rdi1p fusion proteins. 30µg of total protein from BJ5459 cells expressing the indicated proteins were resolved on 13% SDS-PAGE gels and immunoblotted with anti-GFP α Av antibody. Left lane, size markers in kD.

Figure 10: BiFC interactions between Cdc42p and Rdi1p.

A. YN-Rdi1p + CC-Cdc42p



YN-Rdi1p + YC-Cdc42p



B. Phase BiFC

BJ5459

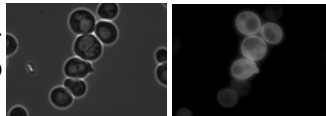
YN-Rdi1p

CC-Cdc42p

YC-Cdc42p

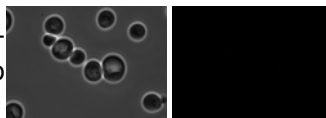
C. Phase BiFC

YN-Rdi1p +
CC-Cdc42-F28Lp



YN-Rdi1p +
CC-Cdc42-D118Ap

YN-Rdi1p +
CC-Cdc42-T17Np



D.

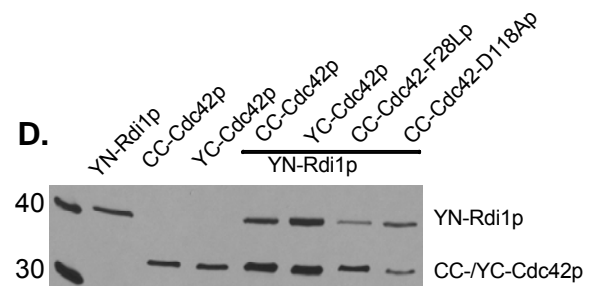


Figure 11. Time-lapse photomicroscopy of Rdi1p-Cdc42p BiFC interactions during the cell cycle. 1607-5D cells expressing YN-Rdi1p and YC-Cdc42p were grown overnight in LF SC –Trp –Ura +Met with 2% galactose + 2% raffinose. Cells were collected by centrifugation, rinsed twice in ddH₂O, and re-suspended in LF SC –Trp –Ura –Met with 2% glucose + 2% raffinose for 3 h at 30°C (>90% cell-cycle arrest as unbudded cells). Cells were collected, rinsed twice in ddH₂O, and 200 µl were re-suspended in LF SC –Trp –Ura –Met with 2% galactose + 2% raffinose to release from cell cycle arrest. Cells were viewed on 1% agar pads made with release media. Numbers indicate time after release from block. **(A)** Arrows represent enhanced BiFC signal at incipient bud sites, the periphery of small- and medium-sized buds, and the mother-bud neck region. **(B)** Cells were grown as in **(A)**. Left, G₁-arrested cells (grown in glucose); Right, cells released from arrest (grown in galactose). Within 5 min of release from G₁ arrest, a ring-like BiFC structure appears (arrows).

Figure 11: Time-lapse photomicroscopy of Rdi1p-Cdc42p BiFC interactions during the cell cycle.

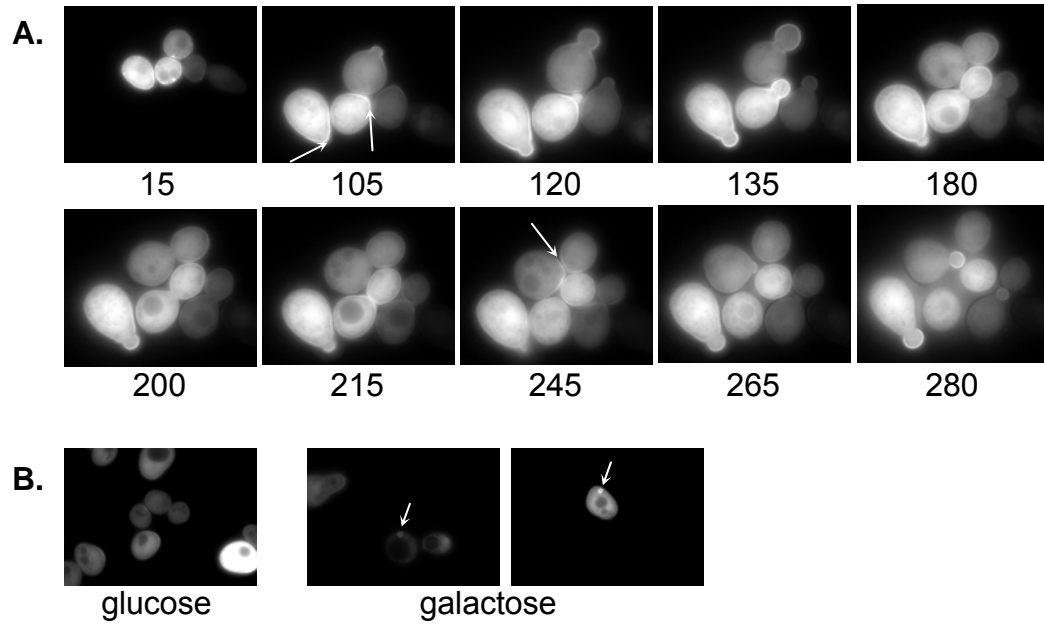


Figure 12. SWISS-MODEL generated structure of a *S. cerevisiae* Cdc42p (blue) - Rdi1p (yellow) complex. Highlighted are the Switch I (lime green) and Switch II (orange) regions of Cdc42p, and the geranylgeranyl moiety (red). Mutated residues are shown in white.

Figure 12: SWISS-MODEL generated structure of a *S. cerevisiae* Cdc42p (blue) - Rdi1p (yellow) complex.

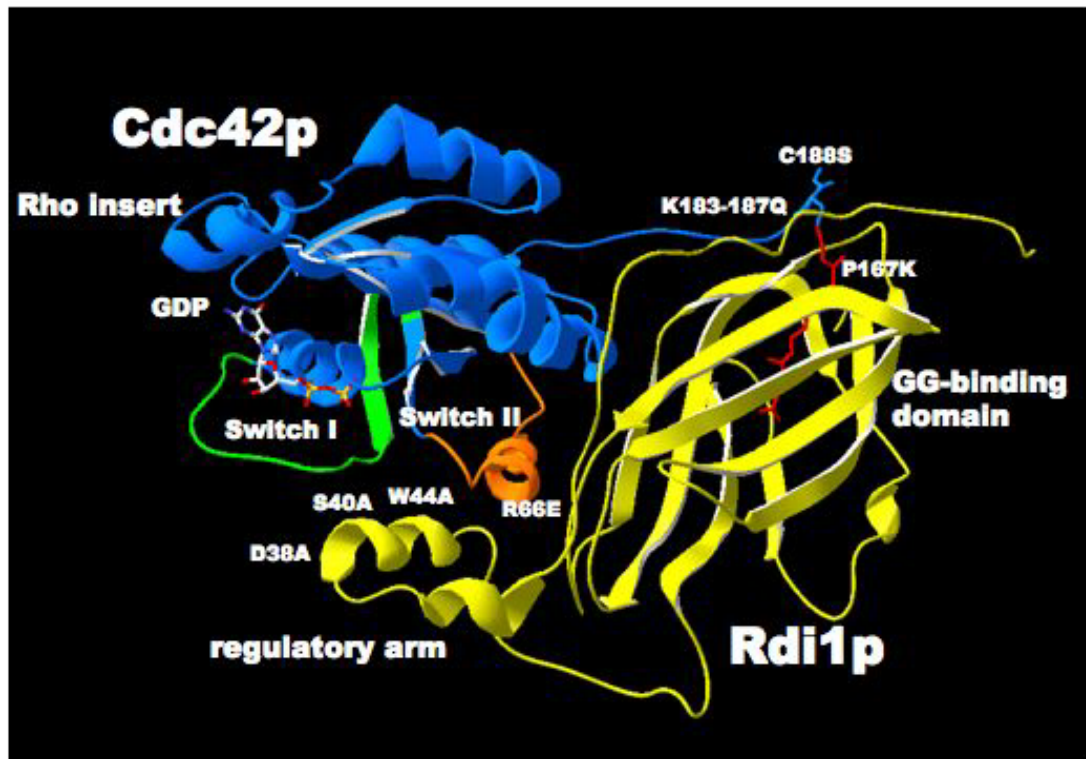


Figure 13. Analysis of mutations affecting the Switch I/Switch II-regulatory arm interactions and geranylgeranyl binding. BiFC interactions were observed as described in Figure 1. **(A)** BJ5459 cells expressing YN-Rdi1p and either CC-Cdc42-R66Ep, CC-Cdc42-K183-187Qp, or CC-Cdc42-K183-187Q,R66Ep. For cells expressing YN-Rdi1p and CC-Cdc42-K183-187Q,R66Ep, both Differential Interference Contrast (left) and BiFC fluorescence (right) photomicrographs are shown to highlight the observation that only ~10% of cells exhibit a BiFC signal. Percentage of cells displaying a predominantly membrane (Cdc42-R66Ep) or cytoplasmic (Cdc42-K183-187Qp) BiFC signal are indicated. **(B)** BJ5459 cells expressing YN-Rdi1p + CC-Cdc42-C188Sp (left) or YN-Rdi1-P167Kp + CC-Cdc42p (right). Far right, predicted structural effect of the P167K mutation. P and mutant K residues are shown in white; geranylgeranyl moiety is shown in red. **(C)** BJ5459 cells expressing YC-Cdc42p and either YN-Rdi1-D38Ap, YN-Rdi1-W44Ap, YN-Rdi1-Δ37-47p, or YN-Rdi1-S40Ap.

Figure 13: Analysis of mutations affecting the Switch I/Switch II-regulatory arm interactions and geranylgeranyl binding.

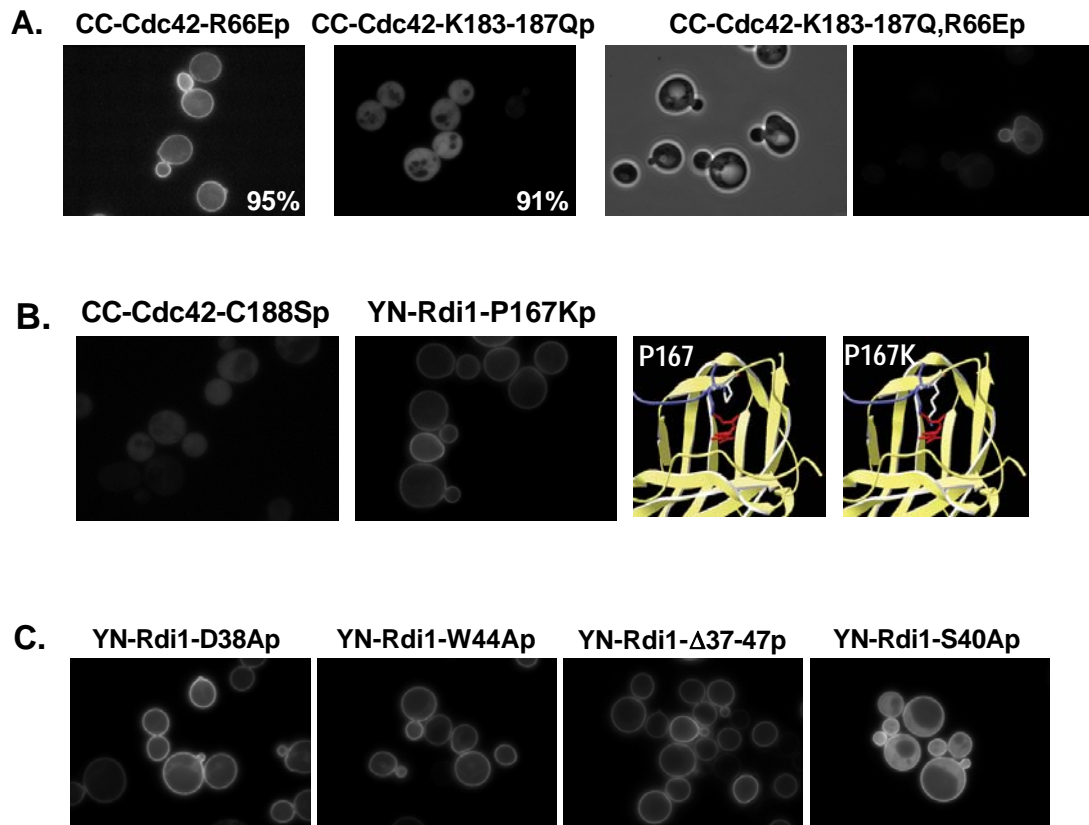
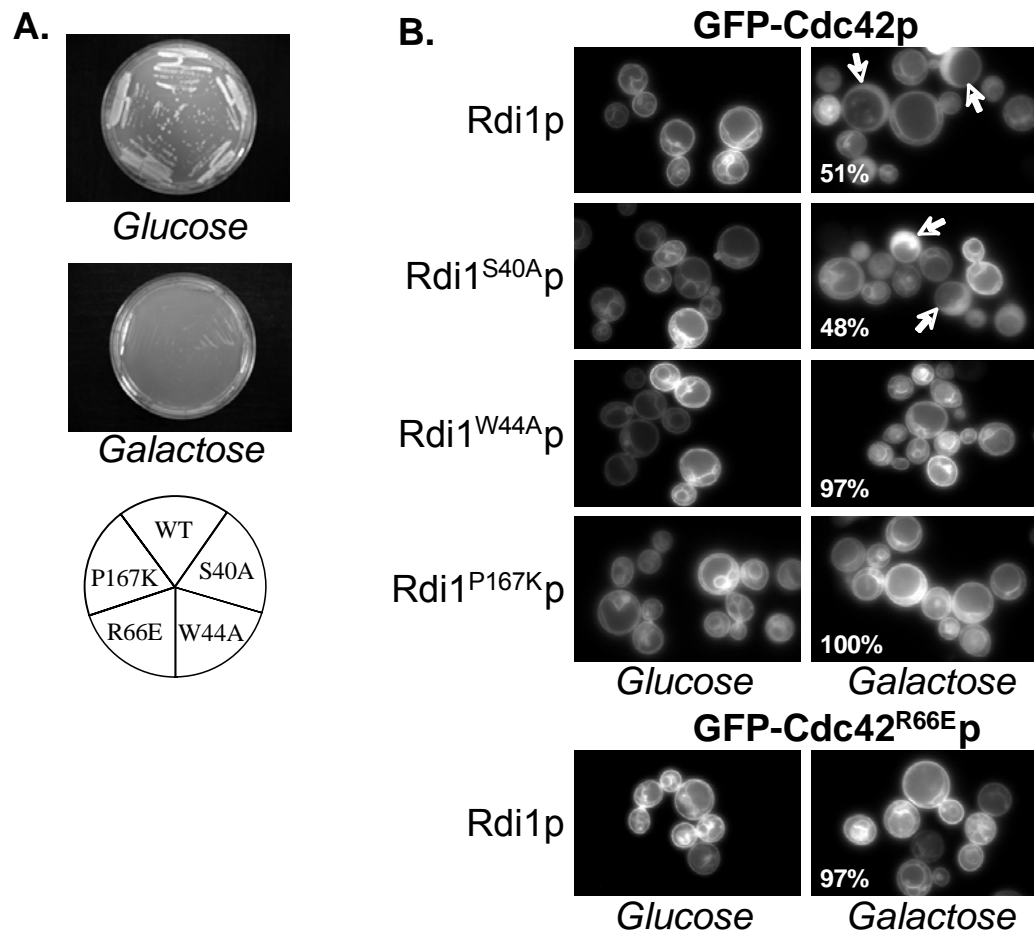


Figure 14. Growth inhibition (**A**) and membrane extraction (**B**) assays with Cdc42p and Rdi1p mutants. (**A**) BJ5459 cells expressing either wild-type Rdi1p, Rdi1(S40Ap), Rdi1(W44Ap), Rdi1(P167Kp), or Cdc42(R66Ep) were grown on repression (glucose) and derepression (galactose) media. (**B**) Top, BJ5459 cells expressing wild-type GFP-Cdc42p and either wild-type Rdi1p or the indicated mutant Rdi1p were grown in LF SC –Ura –Leu –Met with 2% raffinose and either 2% glucose (left) or 2% galactose (right) for 6 h and then observed by fluorescence microscopy. Arrows indicate cells with predominantly cytoplasmic GFP-Cdc42p localization. Percentage of cells [n=100 for Rdi1(P167Kp); n=300 for Rdi1(S40Ap) and Rdi1(W44Ap)] with predominantly membrane GFP-Cdc42p localization are indicated. Bottom, BJ5459 cells expressing GFP-Cdc42(R66Ep) and wild-type Rdi1p were grown and observed as above.

Figure 14: Growth inhibition (A) and membrane extraction (B) assays with Cdc42p and Rdi1p mutants.



CHAPTER 3

POLARIZED TARGETING AND MAINTENANCE OF THE SACCHAROMYCES CEREVISIAE RHOGEF CDC24P DEPENDS ON BOI2P, ENT2P, TOS2P, HUA1P, AND PHOSPHORYLATION OF CDC24P

My contributions to this chapter include experimental design, execution and writing.

Polarized targeting of the *Saccharomyces cerevisiae* RhoGEF

**Cdc24p depends on Boi2p, Ent2p, Tos2p, Hua1p, and
phosphorylation of Cdc24p**

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Running Title: Targeting and maintenance of the RhoGEF Cdc24p

Keywords: Cdc24p, Boi2p, Ent2p, Tos2p, Hua1p, cell-cycle phosphorylation

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Summary

The *Saccharomyces cerevisiae* Rho GTPase Cdc42p is activated at sites of polarized growth (*i.e.*, incipient bud sites, tips of small buds, and the mother-bud neck region) by the precise targeting of its guanine nucleotide exchange factor (GEF) Cdc24p to these sites. Cdc24p is targeted to polarized growth sites at specific times during the cell cycle, as well as being present in the nuclei of G₁-phase cells and post-anaphase mother and daughter cells. This study examines the protein(s) and regulatory mechanisms that function in targeting and maintenance of Cdc24p at polarized growth sites. Localization of the GFP-tagged targeting and anchoring domain of Cdc24p (GFP-Cdc24-647-854p) was assayed in haploid deletion mutants of proteins previously identified as interacting with, or in a complex with, Cdc24p. The *boi2Δ*, *ent2Δ*, *hua1Δ*, and *rsc2Δ* mutants lacked proper localization of GFP-Cdc24-647-854p to sites of polarized growth. In addition, overexpression of Boi2p led to cell death with a defect in GFP-Cdc24p localization. The *skg6Δ* and *tos2Δ* single mutants had properly localized GFP-Cdc24-647-854p, but *skg6Δ tos2Δ* double mutants displayed abnormally localized GFP-Cdc24-647-854p to the mother-bud neck region in pre-anaphase cells. The same abnormal localization pattern was seen when potential cyclin-dependent kinase phosphorylation sites (Ser697 and Thr704) within the Cdc24p targeting domain were mutated to non-phosphorylatable Ala residues and with GFP-Cdc24-Y200Fp. Immunoblots of 2-D polyacrylamide gels indicated that the Ser697 residue is phosphorylated *in*

vivo. Taken together, these data support roles for Boi2p, Ent2p, Hua1p, Rsc2p, Tos2p, and for Cdc24p phosphorylation in targeting and anchoring Cdc24p at sites of polarized growth.

Introduction

Polarization of the actin cytoskeleton into the *S. cerevisiae* bud is coordinated with the cell cycle to ensure the fidelity of mitotic spindle orientation and organelle inheritance and to target secretory vesicles to discrete sites on the plasma membrane for delivery of cell wall remodeling enzymes and new membrane to facilitate apical bud growth [reviewed in (237)]. At the center of these processes is the Rho GTPase Cdc42p [reviewed in (145, 228)]. Cdc42p localizes around the entire plasma membrane (247), but is only active (GTP-bound) at sites of polarized growth. Spatial and temporal control of Cdc42p occurs through localization of, and interaction with, its regulatory proteins. Cdc42p is negatively regulated by several GTPase-activating proteins (GAPs) and a guanine nucleotide dissociation inhibitor (GDI), and activated by its guanine nucleotide exchange factor (GEF) Cdc24p [reviewed in (145, 228)].

Cdc24p is a peripheral membrane protein that is targeted to sites of polarized cell growth (*i.e.*, plasma membrane at incipient bud sites, tips of small buds, and the mother-bud neck region in large-budded cells) at specific times in the cell cycle as well as to the nuclei of unbudded G₁-phase cells and mother and daughter cells post-anaphase (42, 212, 293). Regulation of Cdc24p function is mediated through nuclear sequestration in G₁-phase cells, cell cycle-dependent recruitment to the incipient bud site post-START, anchoring within a multi-protein complex, and amplification and abrogation of activity through feedback loops

[reviewed in (107)]. These regulatory mechanisms appear to operate, in part, through phosphorylation of Cdc24p, and other polarity establishment proteins, which affect their targeting to polarized growth sites in a cell cycle-dependent manner. Phosphorylation of Cdc24p depends on the cyclin-dependent kinase (CDK) Cdc28p (34, 106, 190), the cyclin Cln2p (106, 190), the p21-activated kinase (PAK) Cla4p (34, 106), and the Bem1p scaffold protein (34, 42, 106). Phosphorylation of Boi1p and Boi2p, two proteins that interact with Cdc42p (22) and Cdc24p (90) respectively, facilitates their targeting to the incipient bud site (190). Tos2p, which has a role in anchoring Cdc24p to the bud tip (294), and its homolog Skg6p, are also *in vitro* targets of the Cdc28p-Cln2p CDK complex (300).

A 56-amino acid domain at the carboxyl-terminus of Cdc24p (aa 647-703) was found to be necessary and sufficient for targeting of Cdc24p to the plasma membrane at incipient bud sites, tips of small buds, and the mother-bud neck region in large-budded cells (294). After specific targeting to sites of polarized growth, Cdc24p must be anchored at these sites, which requires the remaining carboxyl-terminal amino acids (aa 704-854) (294). The Cdc24-647-854p 'targeting and anchoring' domain is proposed to be in a multi-protein complex at bud tips with the Cdc24p-interacting proteins Bem1p, Rsr1p, and Tos2p (294).

In this study, we assayed the localization of GFP-Cdc24-647-854p in several haploid deletion mutants of known Cdc24p-interacting proteins. We identified five mutants that displayed abnormal Cdc24p targeting, suggesting that

these proteins, Boi2p, Ent2p, Hua1p, Rsc2p, and Tos2p, have a previously unrecognized role in regulating Cdc24p polarized localization. We have also identified key amino acid residues within the Cdc24p targeting and anchoring domain and CH domain that function in the localization of Cdc24p, and its regulation through phosphorylation.

Materials and Methods

Reagents, media and strains. *S. cerevisiae* strains (listed in Table 1) were grown in synthetic complete (SC) medium with 2% raffinose + either 2% glucose (repressing) or 2% galactose (de-repressing)+ for *GAL1*-promoter induction.

Fluorescence microscopy. Strains transformed with the indicated plasmids were grown in the appropriate low-fluorescence (LF) (272) SC liquid medium to mid-log phase, collected by centrifugation, sonicated and viewed using differential interference contrast optics. An E400 Nikon microscope (Omega Optical, Brattleboro, VT) utilizing an Omega XF100 optical filter cube for GFP-tagged proteins was used for fluorescence microscopy. Small-budded cells (n=100) expressing GFP-Cdc24p, GFP-Cdc24-Y200Fp, GFP-Cdc24-647-854p (and mutations within this domain) were quantified independently by two people to determine the percentage of cells with abnormal localization pre-anaphase at the mother-bud neck region. The percentages reported are the average of the two independent quantifications, which were within +/- 5% of each other. Photomicrograph collages were prepared in Adobe Photoshop CS.

Protein and immunoblot analysis. Total protein lysates were harvested in NP-40 lysis buffer as previously described (34), except that 1X Complete EDTA-free protease inhibitor cocktail (Roche Pharmaceuticals, Nutley, NJ) was used instead of PMSF and leupeptin. Protein concentration was determined by the Bradford

method (Bio-Rad Laboratories, Hercules, CA). A 1:2000 dilution of mouse α -GFP antibody (Roche Pharmaceuticals) and a 1:5000 dilution of goat α -mouse horseradish peroxidase (HRP) (Sigma, St. Louis, MO) were used to detect GFP-fusion proteins.

For immunoprecipitation experiments, 1 mg of protein lysate was incubated for 1 h at 4°C with 10 μ l monoclonal mouse anti-GFP antibodies (Roche Pharmaceuticals) in an additional 400 μ l of NP-40 lysis buffer. 50 μ l of Protein-G Agarose (Roche Pharmaceuticals) was added and incubated overnight at 4°C, and then centrifuged for 20 sec. at 4°C. The supernatant was discarded and the mouse anti-GFP-Protein-G agarose complexes were washed 1X in NP-40 lysis buffer, 1X in 50mM Tris pH 7.5, 500mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate, 1X in 10 mM Tris pH 7.5, 0.1% NP-40 0.05% sodium deoxycholate, followed by two washes in 10 mM Tris pH 7.5. All washes were 10 min. at 4°C. Samples treated with CIP were suspended in 100 μ l of 1X NEB#3 (New England Biolabs, Ipswich, MA) with 10U of calf alkaline phosphatase (New England Biolabs) for 2 hours at 37°C and washes above were repeated. 200 μ l of ASB-14 Buffer (7M urea, 2M thiourea, 2% ASB-14, 0.5% TritonX-100, 20mM DTT) was added for 20 min. at RT with agitation to elute mouse anti-GFP-Cdc24p complexes, and then 0.2% 3/10 ampholyte (Bio-Rad Laboratories) and 0.0002% bromophenol blue was added. The Protein-G agarose was centrifuged for 20 sec. at 4°C and the supernatant transferred to a new tube, which was spun at 14,000 rpm at 25°C for 1 min. to remove any insoluble protein. 185 μ l of the

supernatant was used to actively hydrate the 11 cm ReadyStrip IPG 5-8 (Bio-Rad Laboratories) for 12-15 h, followed by 2-D electrophoresis according to manufacturer instructions in the PROTEAN IEF System (Bio-Rad Laboratories). Isoelectric focusing conditions were as follows: (rapid ΔV) S1 250V for 15 min., S2 8000V 1 hr., S3 8000V for 20000 vhours, S4 500V hold. IPG strips were frozen at -20°C until the 10% SDS-PAGE was run. The pI of GFP-Cdc24-647-854p (6.22) was predicted from the EMBL WWW Gateway to Isoelectric Point Service (<http://www.emblheidelberg.de/cgi/pi-wrapper.pl>).

Results

A screen of haploid deletion mutants identifies proteins involved in targeting of GFP-Cdc24-647-854p to sites of polarized growth

In an asynchronous culture of wild-type cells, ~30% of cells exhibit polarized targeting of Cdc24p, specifically to incipient bud sites in G₁-phase cells, the tips of small-budded cells, and the mother-bud neck region post-anaphase (42, 212, 247, 293). The carboxyl-terminal amino acids 647-854 of Cdc24p are necessary and sufficient to target and anchor Cdc24p at these sites of polarized growth (294). The hypothesis that Cdc24p-interacting protein(s) are involved in this targeting was tested using a series of haploid deletion mutants (Table 10). *boi1Δ*, *ent1Δ*, and *skg6Δ* mutants, although not known to interact with Cdc24p, were also tested in this screen because their homologues did interact with Cdc24p.

GFP-Cdc24-647-854p localized to incipient bud sites, tips of small-budded cells, and the mother-bud neck region in seven of the 11 haploid deletion mutants tested (Table 11). The *air1Δ*, *akr1Δ*, *boi1Δ*, *ent1Δ*, *she2Δ*, *skg6Δ*, and *tos2Δ* mutants displayed wild-type GFP-Cdc24-647-854p localization to polarized growth sites in ~30% of cells in an asynchronous population. However, the *boi2Δ*, *ent2Δ*, *hua1Δ* (Fig. 15A), and *rsc2Δ* (data not shown) mutants displayed wild-type GFP-Cdc24-647-854p localization in <5% of cells in an asynchronous

population. Interestingly, full length GFP-Cdc24p displayed wild-type localization in the *ent2Δ* and *hua1Δ* mutants, with *hua1Δ* mutants also exhibiting punctate cortical localization (Fig. 15B).

Because the *boi2Δ* and *ent2Δ* mutants displayed abnormal localization of GFP-Cdc24-647-854p, their homologues were also examined for a targeting defect. The Ent2p homolog Ent1p and the Boi2p homolog Boi1p have not been previously identified as Cdc24p-interacting proteins, although Boi1p co-immunoprecipitates in a complex with Cdc24p, Boi2p, and Rga2p (190). Both the *boi1Δ* and *ent1Δ* mutants displayed wild-type localization of GFP-Cdc24-647-854p (Fig. 15C), suggesting that Boi1p and Ent1p are not functionally redundant with Boi2p and Ent2p in their ability to target GFP-Cdc24-647-854p. Interestingly, α -factor-induced cell-cycle arrest resulted in proper localization of GFP-Cdc24-647-854p to mating projections in *boi2Δ* and *ent2Δ* mutants (data not shown), indicating that the mechanism for targeting and anchoring Cdc24p at sites of polarized growth is different during mating and mitotic growth.

Tos2p was previously posited to function in a multi-protein complex, containing the Bem1p scaffold protein and the Rsr1p/Bud1p GTPase, to anchor GFP-Cdc24-647-854p at polarized growth sites (294). Tos2p shares 35% amino acid sequence homology with Skg6p, predominantly found within their carboxyl-termini. Skg6p, while not previously shown to interact with Cdc24p, was also examined for a targeting defect. The *tos2Δ* and *skg6Δ* single mutants exhibited wild-type GFP-Cdc24-647-854p localization at polarized growth sites (Fig. 16A;

Table 11). In the *tos2Δ skg6Δ* double mutant, GFP-Cdc24-647-854p localized to incipient bud sites in G₁-phase cells and the mother-bud neck region at cytokinesis; however, 13% of small-budded cells had GFP-Cdc24-647-854p aberrantly localized at the mother-bud neck region in pre-anaphase cells (Fig. 16A). This result suggests that Tos2p and Skg6p share a role in anchoring Cdc24p at the enlarging bud tip prior to the apical-isotropic switch in G₂ phase.

Localization of putative CDK phosphorylation site mutants

Cdc24p has six consensus CDK phosphorylation site sequences (S/T-P-X-K/R). Three of these sites (Ser697, Thr704, and Ser811) are located within the targeting and anchoring domain. Cdc28p, the primary CDK responsible for cell cycle transit, has been implicated in the maintenance of bud growth (190) and in the phosphorylation of Cdc24p (34, 106, 190). The Ser697 and Thr704 amino acids were mutated to nonphosphorylatable Ala residues and the phosphomimetic Glu residues in the context of full-length GFP-Cdc24p. GFP-Cdc24-S697Ap displayed wild-type localization to incipient bud sites and the mother-bud neck region post-anaphase, but in 31% of small-budded cells, GFP-Cdc24-S697Ap localized aberrantly at the mother-bud neck region in pre-anaphase cells (Fig. 16B), similarly to the localization pattern observed with the *tos2Δ skg6Δ* double mutant expressing GFP-Cdc24-647-854p. GFP-Cdc24-S697Ep, GFP-Cdc24-T704Ap and GFP-Cdc24-T704Ep all exhibited wild-type localization to incipient bud sites, tips of small-budded pre-anaphase cells, and

the mother-bud neck region in post-anaphase cells (Fig. 16B). These mutant phenotypes suggest that potential phosphorylation of Ser697 plays a positive role in anchoring Cdc24p at the tips of enlarging buds, possibly through an interaction with Tos2p.

GFP-Cdc24-647-854-S697Ap displayed wild-type localization to incipient bud sites and the mother-bud neck region post-anaphase, but in 40% of small-budded cells, localized aberrantly at the mother-bud neck region in pre-anaphase cells (data not shown). Localization of GFP-Cdc24-647-854-T704Ap was the same as GFP-Cdc24-647-854-S697Ap; however, only 10% of small-budded cells exhibited abnormal localization at the mother-bud neck pre-anaphase (data not shown). 74% of cells expressing GFP-Cdc24-647-854-S697A, T704Ap exhibited abnormal localization at the mother-bud neck pre-anaphase (Fig. 16D).

The mammalian Rho GEF Vav is auto-inhibited through an interaction with the DH domain that is relieved by phosphorylation of the CH domain Tyr174 by Src kinase (4). An equivalent Tyr residue within the CH (Y200) domain of Cdc24p was mutated to a nonphosphorylatable Phe. GFP-Cdc24-Y200Fp displayed wild-type localization to incipient bud sites and the mother-bud neck region post-anaphase, but in 42% of small-budded cells, localized aberrantly at the mother-bud neck region in pre-anaphase cells (Fig. 16E).

Overexpression of Boi2p is lethal and results in depolarization of GFP-Cdc24p

Cells overexpressing Boi1p or Boi2p arrest as large, round, unbudded cells (189), and overexpression of Boi2p from a galactose-inducible promoter in BY4741 cells was lethal (Fig. 17A). This loss-of-polarity phenotype is reminiscent of *cdc24* loss-of-function (280) and overexpression (331) and therefore, may be due to defects in polarized targeting of Cdc24p. To test this hypothesis, Boi2p was overexpressed under the control of a galactose-inducible promoter in BY4741 cells that also expressed GFP-Cdc24p or GFP-Cdc24-647-854p. In glucose-containing media, GFP-Cdc24p and GFP-Cdc24-647-854p exhibited wild-type targeting to incipient bud sites, tips of small-budded cells, and to the mother-bud neck region post-anaphase (Fig. 17B). In galactose-containing media, GFP-Cdc24p and GFP-Cdc24-647-854p still targeted to sites of polarized growth; however in ~ 50% of unbudded cells, GFP-Cdc24-647-854p was diffusely localized throughout the cytoplasm and formed punctate aggregates within the cell. Taken together, these results suggest that overexpression of Boi2p causes a cell-cycle arrest because Cdc24p is not targeted to a discrete site on the plasma membrane.

2-D gel analysis of post-translational modifications to Cdc24-647-854p

In addition to the three consensus CDK phosphorylation sites in Cdc24-647-854p, ELM server analysis of this region also indicated potential consensus phosphorylation sites for casein kinase I and II (Yck1-2p), glycogen synthase kinase-3 (Mrk1p and Ygk3p), and protein kinase A (Tpk1p). Five Ser and Thr

residues within this region have also been identified as phosphorylated residues during G₂/M phase (S.P. Gygi, personal communication). Cdc24p has also been identified as a substrate for ubiquitin conjugation (232). 2-D gel analysis of Cdc24p-647-854p was performed, as 1-D gel analysis of this many potential modifications is inadequate.

GFP-Cdc24-647-854p, with and without phosphatase treatment, and GFP-Cdc24-647-854-S697Ap were immunoprecipitated with α -GFP antibodies and subjected to isoelectric focusing and SDS-PAGE (Fig. 18) as described in Materials and Methods. Four isoelectric species were observed for GFP-Cdc24-647-854p (Fig. 18A) and three for GFP-Cdc24-647-854-S697Ap (Fig. 18B), suggesting that the S697A mutation results in the loss of a modified species. GFP-Cdc24-647-854p treated with phosphatase (Fig. 18C) results in the collapse of the isoelectric species into one spot, indicating that the three additional spots seen in GFP-Cdc24-647-854p are the result of phosphorylation.

Discussion

Cdc42p-dependent polarization of the actin cytoskeleton into the emerging bud is coordinated with the cell cycle to ensure the fidelity of mitotic spindle orientation and organelle inheritance, and to target secretory vesicles to discrete sites on the plasma membrane for delivery of cell wall-remodeling enzymes and new membrane components [reviewed in (237)]. To catalyze this asymmetrical Cdc42p activation, the Rho GEF Cdc24p must be specifically targeted only to

sites of polarized growth. Although the Cdc24p-interacting proteins identified herein may have diverse biological functions, their common role in the targeting and maintenance of Cdc24p at these sites may provide insight into maintenance of other asymmetrically targeted proteins in *S. cerevisiae*.

Both overexpression of either Boi1p or Boi2p [Fig. 3; (189)] and *boi1Δ boi2Δ* double mutations led to cell-cycle arrest as large, round, unbudded cells (22, 189), reinforcing their role in regulating the bud emergence process. They were initially identified as Bem1p-interacting homologues (22, 189) that localize to incipient bud sites, the periphery of buds, and the mother-bud neck region in large-budded cells (113), and can be found in the nuclei of some unbudded G₁phase cells (216). The abnormal localization of Cdc24p in *boi2Δ*, but not *boi1Δ*, mutants (Fig. 15), and upon overexpression of Boi2p (Fig. 17) strongly suggests that Boi2p, but not Boi1p, plays an important role in regulating Cdc24p localization. The GFP-Cdc24-647-854p fusion protein was also abnormally localized in a *boi2Δ* mutant and upon overexpression of Boi2p, suggesting that Boi2p interacts with amino acid residues within this targeting and anchoring domain. The carboxyl-terminal PH domain of Boi2p was necessary and sufficient for growth (22, 189), and mutation of this domain prevented localization to the bud, while mutation of the SH3 domain prevented localization to the mother-bud neck region (113). Work is currently underway to determine which of these Boi2p functional domain interacts with the Cdc24p targeting and anchoring domain to mediate Cdc24p localization.

Ent2p and Ent1p are epsins required for endocytosis (314). Mutation of residues within the epsin N-terminal homology (ENTH) domain resulted in endocytosis and actin cytoskeleton defects (314), and mislocalized septins and cytokinesis defects (5). The actin, septin, and cytokinesis defects were independent of the endocytosis defect and may arise because of a decrease in levels of GTP-bound Cdc42p, resulting from an ENTH domain loss of interaction with Cdc42p-specific GAPs (5). In an *ent2Δ*, but not *ent1Δ*, mutant, full-length GFP-Cdc24p was localized normally, but GFP-Cdc24-647-854p was not (Fig. 15). This result indicates that Ent2p, but not Ent1p, was needed specifically for the targeting and/or maintenance of the truncated Cdc24p, but not the full-length Cdc24p.

This raises several possibilities regarding the role of Ent2p in Cdc24p localization. It is possible that the role of Ent2p in Cdc24p localization is being mediated through interactions with another protein, not Ent1p, which interacts with amino-terminal Cdc24p domains. Absence of these Cdc24p domains in truncated GFP-Cdc24-647-854p uncovered a targeting function for Ent2p. Another possibility is that polarized targeting of Cdc24p depends on Ent2p-mediated endocytosis and the truncated GFP-Cdc24-647-854p is more sensitive than full-length GFP-Cdc24p to this endocytosis defect.

Cdc24p is part of the CH3 family of proteins that have a single calponin homology (CH) domain at their amino-terminus (157). Although the CH domain was originally identified in the actin-binding protein calponin, there is no evidence

that single CH domains bind actin (94). The Cdc24p CH domain interacts with the Far1p CDK inhibitor (212) and the Rsr1p/Bud1p GTPase (277). Deletion of the CH domain prevents targeting of Cdc24p to sites of polarized growth (277). Cdc24- Δ CHp was artificially targeted to the plasma membrane with a myristoylation signal, but it was still inactive (277). These observations suggest that the CH domain interacts with other proteins to affect GTPase activation and may be the amino-terminal region responsible for the ability of GFP-Cdc24p to localize at polarized growth sites in *ent2 Δ* mutants. Further experimentation will be necessary to uncover the mechanism by which Ent2p regulates Cdc24p localization.

While the delocalization of GFP-Cdc24-647-854p in the *hua1 Δ* and *rsc2 Δ* mutants (Fig. 15) suggests a role for these proteins in Cdc24p targeting, it is not obvious from the limited data available on these proteins how they may be functioning. Hua1p may be involved in actin patch assembly and has been shown to interact with other proteins involved in actin assembly (75), however, a *hua1 Δ* mutant is viable with no obvious cell polarity phenotypes. Hua1p binds to the WW domain of the ubiquitin ligase Rsp5p and the SH3 domain of the endosomal sorting receptor subunit Hse1p (244). This suggests that Hua1p could facilitate ubiquitination and endocytosis of Cdc24p. Likewise, Rsc2p has not been previously associated with cell polarity, but instead with chromatin remodeling processes important for spore morphogenesis (41). Many *rsc2 Δ* mutants exhibited wide, bent mother-bud necks (data not shown), which

resembled the phenotype of *cdc42*^{V36T} (96), suggesting that Rsc2p may have a role in transcriptional activation of genes essential for vegetative morphogenesis (*i.e.*, septins and Cdc24p targeting and/or maintenance proteins).

The loss of GFP-Cdc24p localization in the *skg6Δ tos2Δ* double mutant, but not the two single mutants, suggests that Skg6p and Tos2p have redundant functions in Cdc24p targeting and/or anchoring in the plasma membrane. However, only Tos2p has been shown to interact with Cdc24p (75) and to function as a membrane anchor for Cdc24p (294). *SKG6* was identified in a screen for suppressors of *kex2Δ gas1Δ* mutant synthetic lethality (296), as a dosage suppressor of *cdc42-118* (75), and, along with *TOS2*, as a dosage suppressor of *gic1Δ gic2Δ* mutants (86), suggesting that it too has a function in cell polarity processes. Both Tos2p and Skg6p are predicted to be integral membrane proteins, with a single transmembrane-spanning domain in their amino-termini, and both localize at incipient bud sites, the bud tip of small- and medium- budded cells, and as a double-ring at the mother-bud neck region in large-budded cells (86, 297). Skg6p and Tos2p are also phosphorylated *in vitro* by a Cdc28p-Clb2p complex (300). It remains to be determined whether or not Tos2p is the physiologically relevant membrane anchor for Cdc24p within the plasma membrane and whether phosphorylation of Cdc24-S697A facilitates its interaction with Tos2p and maintenance at the plasma membrane.

The amino-terminus of the Rho1p GEF, Tus1p, is phosphorylated in G₁ by Cdc28p-Cln2p, leading to activation of Rho1p at incipient bud sites (156).

Hyperphosphorylation of Cdc24p is correlated with high levels of Cln2p in G₁ and inhibition of Cdc28p activity after polarization results in the depolarization of Cdc42p (190). Taken together, this suggests that phosphorylation of Rho GEFs in G₁ by Cdc28p-Cln2p could be a conserved mechanism to target them to incipient bud sites, and to maintain their localization in the growing bud, to activate Rho GTPases.

Two potential Cdc28p phosphorylation sites (S697 and T704) were mutated to nonphosphorylatable Ala residues in this study, and their affect, both alone and in combination, on the localization of Cdc24p was determined. *cdc24(T704A)* had little effect on the localization of GFP-tagged full-length Cdc24p (wild-type targeting) or amino acids 647-854 of Cdc24p (10% abnormal pre-anaphase mother-bud neck region targeting). *cdc24(S697A)* had a greater effect on both full-length Cdc24p and amino acids 647-854 (31% and 40%, respectively, abnormal pre-anaphase mother-bud neck region targeting). The percentage of small-budded cells exhibiting abnormal pre-anaphase mother-bud neck region targeting significantly increased to 74% when both mutations were combined in the targeting domain. Although it is unknown how the abnormal pre-anaphase mother-bud neck region targeting of Cdc24p occurs, one possible explanation is that this represents protein that was not maintained at the tip of the emerging bud (mother localization) and protein that was not maintained at the tip of the growing bud (bud localization). This could suggest that phosphorylation of Ser697 and Thr704 is not essential for localization to the incipient bud site, but is

required for its maintenance at the tip of the growing bud. Because the septin ring that forms at the incipient bud site transforms into a collar as the bud emerges (97), it is also possible that the aberrant mother-bud neck region localization represents protein that was targeted to the incipient bud and then 'split' when the septin collar formed because of an association with the septins.

Currently, research is underway to address several outstanding questions raised by this work. The aberrant localization of GFP-Cdc24p to the mother-bud neck region pre-anaphase is observed in the *tos2Δ skg6Δ* mutant and when three amino acid residues are mutated, S697, T704 and Y200. One possible interpretation of this result is that the S697 and T704 residues, which reside within the targeting domain of Cdc24p, interact with the transmembrane proteins Tos2p and Skg6p, to achieve targeting and maintenance at sites of polarized growth that is phosphorylation-dependent. A yeast-two hybrid experiment is in progress to determine if: (1) Cdc24p interacts with Skg6p, (2) Cdc24-647-854p interacts with Tos2p and Skg6p, and (3) Cdc24-647-854-S697Ap and Cdc24-647-854-S697A, T704Ap exhibit a loss of interaction with Tos2p and Skg6p.

A 2D SDS-PAGE analysis of GFP-Cdc24-T704Ap and GFP-Cdc24-S697A, T704Ap are in progress to determine which isoelectric species are affected by the inability to phosphorylate these residues. 2D SDS-PAGE analysis of GFP-Cdc24-S697Ap indicates that this mutation results in the loss of a species that corresponds to the potential absence of two phosphorylation events. One possible explanation for this observation is that the phosphorylation

of Ser697 is an event that facilitates the phosphorylation of another residue within the targeting and anchoring domain.

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Table 10: *Saccharomyces cerevisiae* strains used in this study.

Strain or ORF	Genotype	Source
BY4741	<i>a his3 leu2 met15 ura3</i>	(297)
RG5874	<i>a his3 leu2 met15 ura3 tos2::G418</i>	Research Genetics
NTY372	<i>a his3 leu2 met15 ura3 tos2::G418 skg6::HIS3</i>	(297)
NTY357	<i>a his3 leu2 met15 ura3 skg6::HIS3</i>	(297)
RG3111	<i>a his3 leu2 met15 ura3 boi1::G418</i>	Research Genetics
YER114cΔ	<i>a his3 leu2 met15 ura3 boi2::G418</i>	Open Biosystems
YDL161wΔ	<i>a his3 leu2 met15 ura3 ent1::G418</i>	Open Biosystems
YLR206wΔ	<i>a his3 leu2 met15 ura3 ent2::G418</i>	Open Biosystems
YIL079cΔ	<i>a his3 leu2 met15 ura3 air1::G418</i>	Open Biosystems
YDR264cΔ	<i>a his3 leu2 met15 ura3 akr1::G418</i>	Open Biosystems
YGR268cΔ	<i>a his3 leu2 met15 ura3 hua1::G418</i>	Open Biosystems
YKL130cΔ	<i>a his3 leu2 met15 ura3 she2::G418</i>	Open Biosystems
YLR357wΔ	<i>a his3 leu2 met15 ura3 rsc2::G418</i>	Open Biosystems

Table 11: Summary of GFP-Cdc24aa647-854p and GFP-Cdc24p localization data for deletion strains tested in this study.

Strain	Protein function	GFP-Cdc24-647-854p	GFP-Cdc24p
<i>air1Δ</i>	mRNA processing	+	NT
<i>akr1Δ</i>	palmitoyl transferase	+	NT
<i>boi1Δ</i>	bud emergence	+	+
<i>boi2Δ</i>	bud emergence	-	-
<i>ent1Δ</i>	endocytosis	+	+
<i>ent2Δ</i>	endocytosis	-	+
<i>hua1Δ</i>	actin patch assembly	-	+* ¹
<i>rsc2Δ</i>	chromatin remodeling	-	NT
<i>she2Δ</i>	mRNA bud restriction	+	NT
<i>skg6Δ</i>	unknown	+	+
<i>tos2Δ</i>	unknown	+	+
<i>tos2Δ skg6Δ</i>	unknown	+* ²	+

+, ~30% of cells from an asynchronous population exhibit wild-type localization to polarized growth sites as previously shown (293, 294);

-, < 5% of cells from an asynchronous population exhibit wild-type localization;

+*¹, punctate cortical localization in addition to wild-type localization;

+*², pre-anaphase localization to the mother-bud neck region in ~ 13% of small-budded cells (n=85 cells) in addition to wild-type localization;

NT, not tested

Figure 15. *boi2Δ*, *ent2Δ*, and *hua1Δ* mutants do not target GFP-Cdc24-647-854p to sites of polarized growth.

(A) *boi2Δ*, *ent2Δ*, and *hua1Δ* mutants expressing GFP-Cdc24-647-854p were grown in LF SC medium (- Leu, 0.1X Met) to mid-log phase and observed by fluorescence microscopy. Arrows indicate wild-type polarized targeting of GFP-Cdc24-647-854p to the tip of a small-budded cell and to the mother-bud neck region in a post-anaphase cell (as determined by GFP fluorescence in both mother and daughter nuclei). The *ent2Δ* and *hua1Δ* mutants have diffuse cytoplasmic and nuclear localization of GFP-Cdc24-647-854p, but no polarized targeting.

(B) *boi2Δ*, *ent2Δ*, and *hua1Δ* mutants expressing GFP-Cdc24p were grown and observed as described above. Most *boi2Δ* mutants have diffuse cytoplasmic and nuclear localization of GFP-Cdc24p, and little polarized targeting. Arrows indicate wild-type polarized targeting of GFP-Cdc24p as in (A). The asterisks (*) marking *hua1Δ* mutant cells indicate punctate cortical localization.

(C) *boi1Δ* and *ent1Δ* mutants expressing GFP-Cdc24-647-854p were grown and observed as described above. Arrows indicate wild-type polarized targeting of GFP-Cdc24p as in (A).

Figure 15. *boi2* Δ , *ent2* Δ , and *hua1* Δ mutants do not target GFP-Cdc24-647-854p to sites of polarized growth.

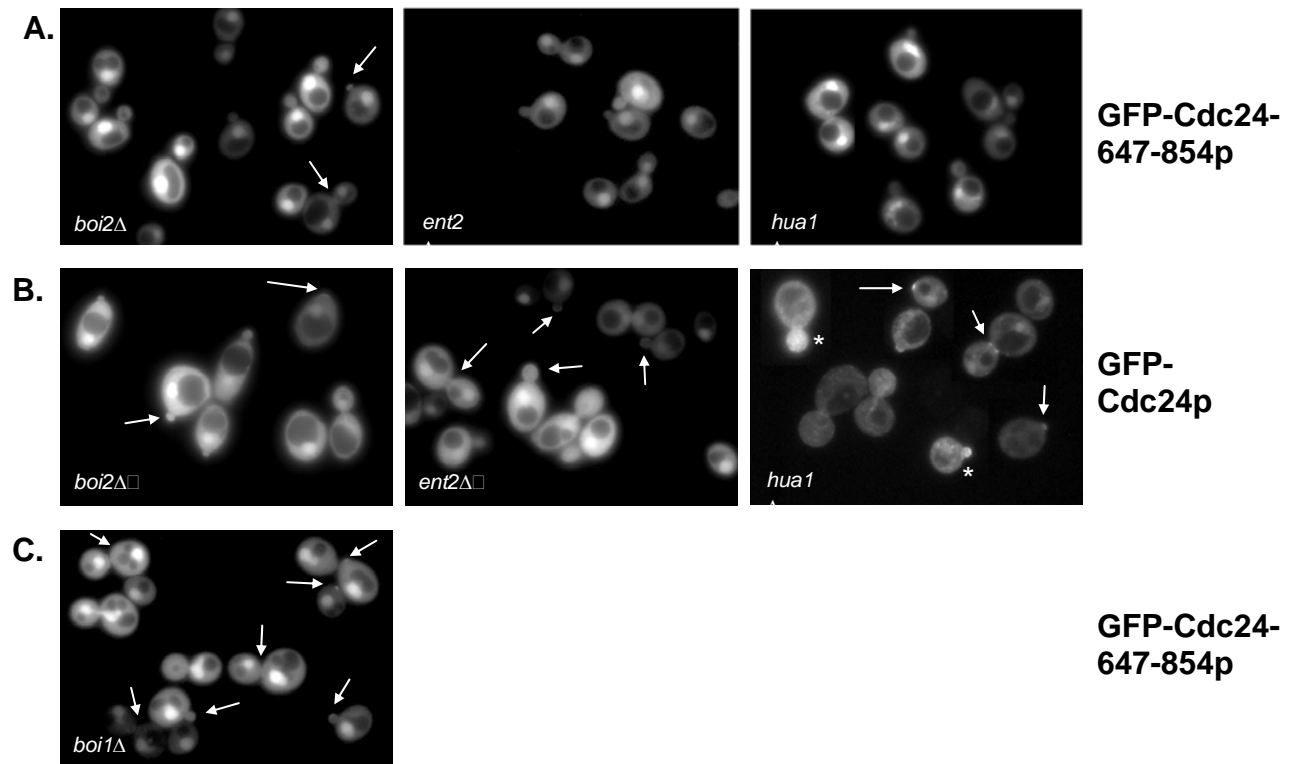


Figure 16. Aberrant targeting of GFP-Cdc24-647-854p and mutations within this domain, GFP-Cdc24-S697Ap, GFP-Cdc24-S697A, T704Ap and GFP-Cdc24-Y200Fp to the mother-bud neck region in pre-anaphase wild-type and mutant cells.

(A) *skg6Δ* and *tos2Δ* single mutants, and *skg6Δ tos2Δ* double mutants expressing GFP-Cdc24-647-854p were grown in low-fluorescent SC medium (-Leu, 0.1X MET) to mid-log phase and observed by fluorescence microscopy. Arrows indicate wild-type polarized targeting of GFP-Cdc24-647-854p to the tips of small-budded cells and the mother-bud neck region in post-anaphase cells. The asterisks (*) marking the *skg6Δ tos2Δ* mutant cells indicate small-budded cells with GFP-Cdc24-647-854p abnormally targeted to the mother-bud neck region pre-anaphase.

(B) BY4741 cells expressing GFP-Cdc24-S697Ap, GFP-Cdc24-S697Ep, GFP-Cdc24-T704Ap, and GFP-Cdc24-T704Ep, as indicated, were grown and observed as described above. The arrows and asterisks (*) indicate cells with the same localization patterns as in (A).

(C) BY4741 cells expressing GFP-Cdc24-S697A,T704Ap were grown and observed as described above. The arrows and asterisks (*) indicate cells with the same localization patterns as in (A).

(D) BY4741 cells expressing GFP-Cdc24-647-854-S697A,T704Ap were grown and observed as described above. The arrows and asterisks (*) indicate cells with the same localization patterns as in (A).

(E) BY4741 cells expressing GFP-Cdc24-S697A,T704Ap were grown and observed as described above. The arrows and asterisks (*) indicate cells with the same localization patterns as in (A).

Figure 16. Aberrant targeting of GFP-Cdc24-647-854p and mutations within this domain, GFP-Cdc24-S697Ap, GFP-Cdc24-S697A, T704Ap and GFP-Cdc24-Y200Fp to the mother-bud neck region in pre-anaphase wild-type and mutant cells.

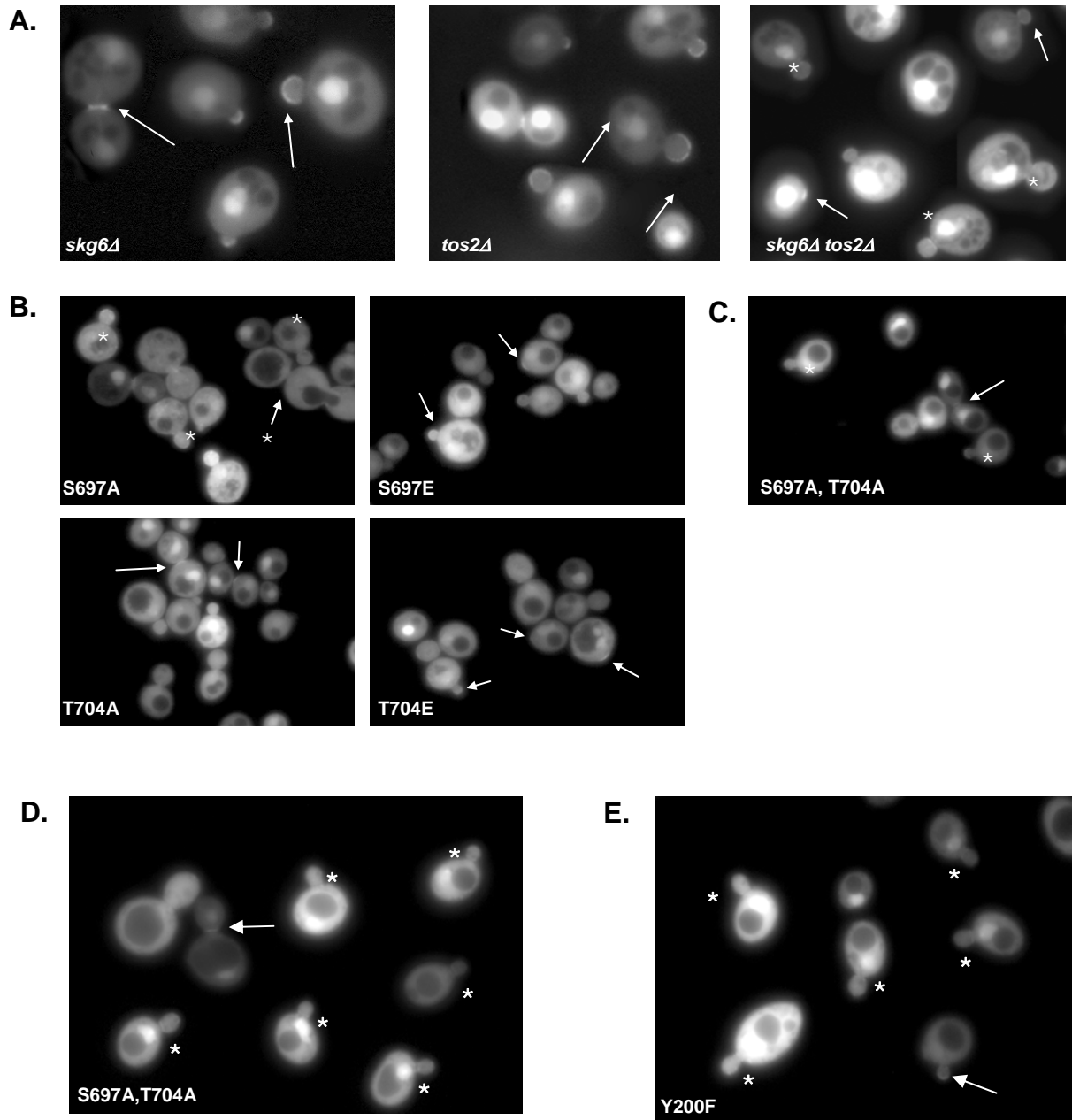


Figure 17: Growth inhibition and loss of polarized Cdc24p upon overexpression of Boi2p.

(A) BY4741 cells containing either a GFP-Cdc24p-expressing plasmid + a *GAL1* control vector (GFP-Cdc24p sector), a *GAL1*-Boi2p-expressing plasmid + a GFP control vector (*GAL1*-Boi2p sector), or *GAL1*-Boi2p-expressing + GFP-Cdc24p-expressing plasmids (*GAL1*-Boi2p, GFP-Cdc24p sector) were grown on repression (SC + raffinose + glucose) and de-repression (SC + raffinose + galactose) media at 30°C.

(B) BY4741 cells containing plasmids expressing the indicated wild-type and mutant proteins were grown under repressing (+ glucose) or de-repressing (+ galactose) conditions in LF SC media (-Ura, -Leu, 0.25X MET) with 2% raffinose for 6 h and then observed by fluorescence microscopy. Arrows indicate cells that have punctate localization of GFP-Cdc24-647-854p within the cytoplasm upon overexpression of Boi2p.

Figure 17. Growth inhibition and loss of polarized Cdc24p upon overexpression of Boi2p.

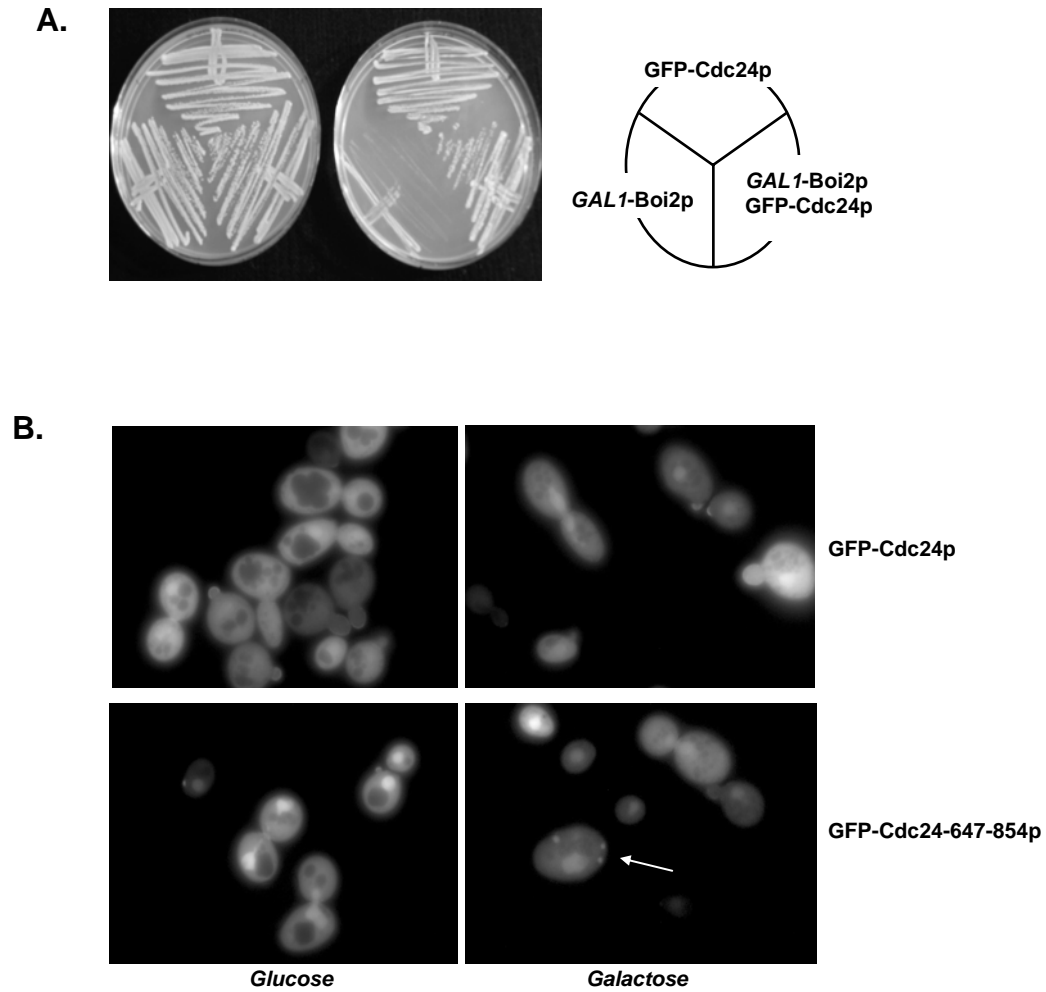
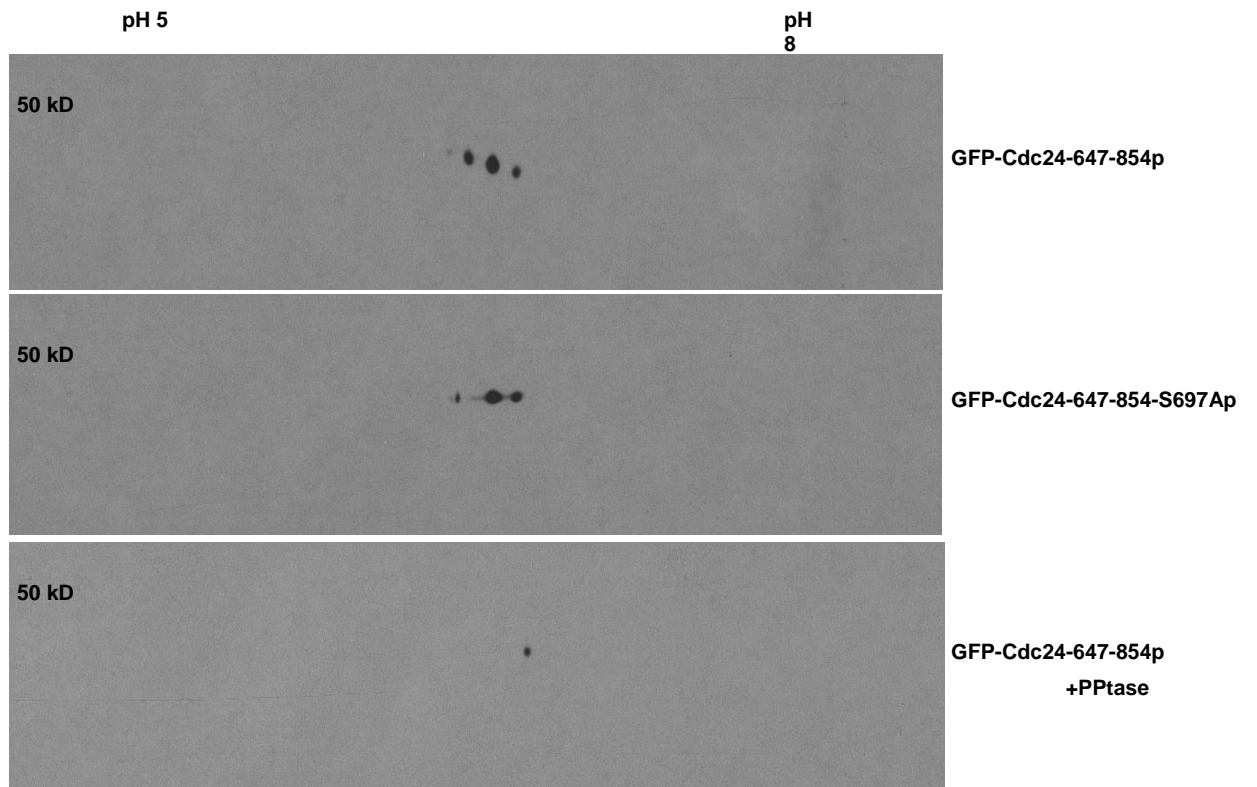


Figure 18. 2-D SDS-PAGE analysis of Cdc24-647-854p.

2-D gel analysis was performed as described in materials and methods and protein was visualized by immunoblotting with α -GFP.

Figure 18. 2-D SDS-PAGE analysis of Cdc24-647-854p.



CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

The local activation of *S. cerevisiae* Rho GTPases is likely achieved by a concerted inactivation of negative regulators (GAPs and GDI) and activation of positive regulators (GEFs) (107). ***The work presented in this dissertation directly contributes to our understanding of Rdi1p-Cdc42p in vivo complex formation and the mechanism of targeting Cdc24p specifically to sites of polarized growth to achieve spatial regulation of Cdc42p.***

Bimolecular Fluorescence Complementation (BiFC)

The technique of bimolecular fluorescence complementation (BiFC) was used to study Rdi1p-Cdc42p *in vivo* complex formation (see **Chapter 2**). In BiFC, the formation of fluorescent complexes from non-fluorescent GFP-variant fragments is facilitated by the interaction of the fused proteins. Unlike fluorescence resonance energy transfer (FRET) [the transfer of excitation energy between two fluorophores], formation of the fluorophore is not exclusively orientation- or distance-dependent; therefore, insertion of a peptide linker (*i.e.*, 8-Ala used in Chapter 2) is generally sufficient to permit fluorophore formation (151). Fluorophore formation is initiated by the interaction of the fused proteins, which is followed by fluorophore maturation ($t_{1/2}$ ~50 minutes), which is essentially irreversible [reviewed in (151)]. Therefore, FRET enables the visualization of immediate protein-protein interactions, while BiFC is useful for steady-state studies. A BiFC-based FRET, which combines the advantages of both

techniques, has been developed to study ternary interactions (278), and BiFC has been combined with flow cytometry to facilitate quantitative analysis and sorting (202).

Regulation of the Cdc42p Polarity Regulator:

The Rdi1p-Cdc42p Complex

The BiFC data presented in **Chapter 2** represents the first comprehensive use of this technique to study *in vivo* protein-protein interactions in *S. cerevisiae*. Rdi1p and Cdc42p interacted throughout the cytoplasm and around the periphery of the cell, and this interaction was enhanced at incipient bud sites, tips of small- and medium-budded cells and at the mother-bud neck. Following release from a G₁-phase cell cycle arrest, the Rdi1p-Cdc42p complex forms a transient, ring-like structure. Amino acid residues were mutated that were predicted, based on a homology-modeled structure, to result in disrupted complex formation. The BiFC data from these mutants showed that the Rdi1p-Cdc42p complex could be shifted to the plasma membrane or the cytosol. Overexpression of Rdi1p mutants indicated that complex located exclusively at the plasma membrane was dominant-negative and lethal; indicating that maintenance of a cytosolic pool of Cdc42p is essential (see **Targeted Vesicle Delivery**).

The ring-like structure was not seen when studying the localization of Rdi1p (249) or Cdc42p (247) by themselves. There are several possible explanations for this. This transient event was most likely seen because of a

combination of the G₁ arrest, subsequent synchronous release, and the propensity of fluorophore formation for trapping transient interactions. The phenomenon of 'eclipsed distribution' describes how large amounts of dual-targeted proteins at a specific subcellular location can prevent small amounts of protein that are targeted to another location from being seen (242). This is often the case when the non-targeted protein in the cytosol (*i.e.*, Rdi1p or Cdc42p) obscures its counterpart that is targeted to a specific subcellular location. BiFC can overcome this problem because the large amount of non-targeted protein in the cytosol is invisible unless it interacts with its partner.

The composition of the ring is intriguing and is still unknown. It is possible that the Rdi1p-Cdc42p ring-like complex co-localizes with the septins, which coalesce into a ring ~15 minutes before the bud emerges (97). Septin ring assembly requires Cdc42p GTP-GDP cycling (95); therefore, it is possible that the ring-like complex is Rdi1p-dependent delivery and/or removal of Cdc42p from the cortex where the septin ring is being assembled. The GTP-GDP cycling requirement would also dictate that GAPs and Cdc24p also be in the ring, although this is currently not known. Time-lapse photomicroscopy of the Rdi1p-Cdc42p BiFC complex immediately following START could determine if the bud forms at the same site as the ring-like complex. Co-localization of the YFP-tagged ring-like complex with CFP-tagged septins, GAPs and Cdc24p could identify other proteins within this structure.

Regulation of the Cdc42p Polarity Regulator:

Rdi1p-dependent Membrane Extraction of Cdc42p

RhoGDI-dependent extraction of Rho GTPases from membranes is proposed to occur in two steps (124, 215). First, the carboxyl-terminal GG-binding domain of RhoGDI interacts with Cdc42, followed by binding of the amino-terminal regulatory arm to the Cdc42 Switch I and II regions. The Cdc42 geranylgeranyl moiety is then inserted into the GG-binding domain, facilitated by interaction between the Cdc42 carboxyl-terminal poly-lysine region and the GDI acidic patch. The BiFC data indicates that mutations in the Rdi1p regulatory arm or in the GG-binding domain are insufficient to prevent Rdi1p-Cdc42p complex formation; however, mutations in these two domains can prevent extraction of Cdc42p from the plasma membrane. This indicates that both domains act cooperatively to bind to and extract Cdc42p from the plasma membrane.

Overexpression of Rdi1p results in inhibition of cell growth and loss of GFP-Cdc42p membrane localization (155, 188, 249). Overexpression of *rdi1* regulatory arm or GG-binding domain mutants did inhibit cell growth, but did not result in the redistribution of GFP-Cdc42p from the plasma membrane to the cytoplasm. This suggests that the dominant-negative interaction of the *rdi1* mutants with Cdc42p at the plasma membrane could be interfering with Cdc42p-effector protein interactions, GDP-GTP exchange, and/or extraction and redelivery to the plasma membrane. Although *RDI1* is not essential, this work indicates that Rdi1p does functionally interact with Cdc42p throughout the cell cycle at sites of polarized growth to affect delivery and extraction of Cdc42p at

the plasma membrane and that perturbation of this interaction can have significant consequences.

Regulation of the Cdc42p Polarity Regulator:

Regulation of Rdi1p-Cdc42p Complex Formation

The *RDI1* transcript is not cell cycle-regulated [SGD, (284) (56)]. Time-lapse photomicroscopy of Rdi1p-Cdc42p interactions during the cell cycle (see **Chapter 2**) indicates that complex formation is regulated in a cell cycle-dependent manner. This regulation likely occurs through reversible posttranslational modifications (*i.e.*, phosphorylation, acetylation, methylation and ubiquitination). These posttranslational modifications, which can occur alone or in combination and can affect complex formation by creating or abolishing binding sites [reviewed in (132)].

Three Phos-Arg molecular switches (Ser101, Tyr144 and Ser148, and Ser174) regulate complex formation between mammalian RhoGDI and Cdc42 (104). Phos-Arg switches consist of phosphorylatable residues followed by Arg, which can be dimethylated. Phos-Arg switches exhibit negative crosstalk (132), since phosphorylation and methylation are mutually exclusive events. Ser101 and Ser174 are phosphorylated by Pak-1, resulting in the dissociation of the RhoGDI α -Rac complex (70); however, neither PAK site, or the Tyr144 and Ser148 Phos-Arg switch, are conserved in Rdi1p. ELM analysis of Rdi1p predicted that Ser180, the yeast equivalent of mammalian Ser174, was a

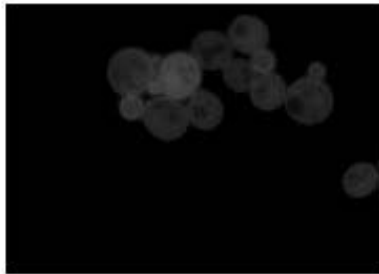
potential Yck1p phosphorylation site. *RDI1* Ser180 was therefore mutated to a non-phosphorylatable Ala and phosphomimetic Glu and analyzed by BiFC for complex formation with Cdc42p (**Figure 19**).

An Rdi1-S180Ep-Cdc42p wild-type BiFC signal was observed in the cytoplasm and around the periphery of the cell at the plasma membrane, and was enhanced at sites of polarized growth (**Figure 19A**). An Rdi1-S180Ap-Cdc42p BiFC signal was observed only in the cytoplasm (**Figure 19B**). These results suggest that an Rdi1p-Cdc42p BiFC signal in an *S. cerevisiae* strain with a deletion of the kinase responsible for this phosphorylation should phenocopy the Rdi1-S180Ap-Cdc42p BiFC signal. A wild-type BiFC signal is observed in *cla4Δ* (**Figure 19C**) and *skm1Δ* (**Figure 19D**) mutants, indicating that Ser180 is not phosphorylated by these PAKs. This result could also indicate that PAK-dependent phosphorylation might not play a role in the dissociation of the Rdi1p-Cdc42p complex. Analysis of Rdi1p-Cdc42p complex formation in *yck1Δ yck2^{ts}* mutants (253, 254) should be done to determine if *rdi1(S180)* is phosphorylated by Yck1p or its homologue Yck2p. If a cytoplasmic BiFC signal is observed in *yck1Δ yck2^{ts}* mutants, this would indicate that yeast casein kinase has a role in targeting the Rdi1p-Cdc42p complex to the plasma membrane through phosphorylation of Rdi1p. BiFC analysis of Rdi1p-Cdc42p complex formation in kinase temperature-sensitive strains with potential consensus phosphorylation sites within Rdi1p (*i.e.*, MAPK, GSK-3, PIKK, and PLK) should enhance our

Figure 19: Rdi1p-Cdc42p complex formation can be restricted to the cytoplasm by mutating *rdi1*(S180).

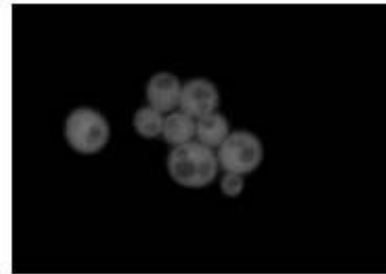
A. BiFC of Ser180 to Glu results in wild-type BiFC. **B.** BiFC of Ser180 to Ala results in BiFC exclusively in the cytoplasm. BiFC between Rdi1p and Cdc42p in **C.** *cla4Δ* and **D.** *skm1Δ* is wild type (C. Morton, K. Cole, D. Johnson, unpublished results).

A. YN-Rdi1-S180Ep + YC-Cdc42p



BJ5459

B. YN-Rdi1-S180Ap + YC-Cdc42p



BJ5459

C. YN-Rdi1p + YC-Cdc42p



cla4Δ

D. YN-Rdi1p + YC-Cdc42p



skm1Δ

understanding of how this complex is regulated in a cell-cycle dependent manner.

Lysine acetylation of RhoGDI K141 blocks its functional interaction with RhoGTPases (152); however, in contrast to phosphorylation and ubiquitination, little is known about its significance. Rdi1p was identified *in vitro* as a potential target of the ubiquitin ligase Rsp5p (164). The recognition sequence of Rsp5p, (L/P)PXY is found once in Rdi1p, ¹⁵⁴KPFY. Rdi1p Tyr157 is also identified by ELM as a potential YXXΦ motif that is recognized by the adaptor protein (AP) complex. Since monoubiquitination of membrane proteins often serves as a signal for endocytosis and subsequent proteasome degradation (119, 120), this may suggest that the addition of ubiquitin to Lys154 might provide a signal for endocytosis of Rdi1p; however, the significance of Lys154 and Tyr157 in the function and localization of Rdi1p is currently unknown.

Tos2p (⁵³⁰KPRY), Skg6p (⁶⁵¹KPSY), and Boi2p (⁴⁴⁴KPPSY), which have roles in the targeting and maintenance of the GEF Cdc24p, also have an Rsp5p recognition site, although they were not identified in the *in vitro* screen. *TOS2*, *SKG6* and *BOI2* transcripts are periodic, in contrast to *RDI1* [SGD, (284) (56)], which suggests that these proteins may be ubiquitinated, endocytosed and targeted to the vacuole in a cell cycle-dependent manner, while the significance of Rdi1p ubiquitination remains unclear. In addition to endocytosis, monoubiquitin also has roles in altering protein function and localization and in

regulating protein-protein interactions [reviewed in (269)]. Ubiquitination of Rdi1p may, therefore, affect its localization and interaction with Rho GTPases.

Regulation of the Cdc42p Polarity Regulator:

Targeting the RhoGEF Cdc24p to Sites of Polarized Growth

Chapter 1 provided background information on the five factors known to contribute to maintenance of polarized cortical domains in *S. cerevisiae* (see **Figure 6**): PIs, mRNA and cER localization, targeted vesicle delivery, diffusion restriction and endocytic recycling. The role of PIs and vesicle transport in the targeting and maintenance of Cdc24p was not directly addressed with the screen of haploid deletion mutants (see **Chapter 3**). Diffusion restriction (*tos2Δ skg6Δ*, *boi2Δ*, *ent2Δ* and *hua1Δ*), mRNA and cER localization (*she2Δ* and *air1Δ*), and endocytosis (*ent2Δ* and *hua1Δ*) were partially addressed by the screen.

The Cdc24p targeting and anchoring domain had wild-type localization at sites of polarized growth in *she2Δ* and *air1Δ* mutants, indicating that mRNA localization to these sites that is dependent on She2p or Air1p is not essential for the targeting and maintenance of Cdc24p. Restricting the diffusion away from sites of polarized growth through anchoring in a multi-protein complex (*tos2Δ skg6Δ*, *hua1Δ* and *boi2Δ*, *ent2Δ* mutants mislocalize or fail to localize, respectively, GFP-Cdc24-647-854p) and endocytosis (*ent2Δ* and *hua1Δ* fail to localize GFP-Cdc24-647-854p) do have a role in the targeting and maintenance of Cdc24p at sites of polarized growth. Research in progress intends to address

further the role that Tos2p, Skg6p, Boi2p, Ent2p and phosphorylation of Cdc24p (see following section) have in the targeting and maintenance of Cdc24p at bud sites.

Regulation of the Cdc42p Polarity Regulator:

The RhoGEF Cdc24p as a Signal Integrator

Mammalian Rho GEFs often become constitutively activated when the amino acids before the DH domain are removed. Although sequence conservation of this region is low, this suggests that intramolecular inhibition of Rho GEFs is highly conserved and an important mechanism of regulation (257). The mammalian Rho GEF Vav, specifically expressed in hematopoietic cells, is auto-inhibited through an interaction with the DH domain that is relieved by phosphorylation of the CH domain Tyr174 by Src kinase (3). However, in *S. cerevisiae*, deletion of the CH domain can't complement a *cdc24Δ* mutant and resulted in a loss of maintenance at bud tips, presumably through a loss of interaction with Rsr1p (277). This indicates that the CH3 domain of Cdc24p could be a signaling integration module, or is essential for its localization to polarized growth sites, without intramolecular inhibitory effects. This is supported by the observation that GFP-Cdc24-Y200Fp is able to complement the *cdc24-4^{ts}* mutant (K. Cole and D. Johnson, unpublished results), indicating that this protein is not maintained in an autoinhibited state (as would be predicted from the Vav

model), because constitutively active *CDC24* should arrest cells as large, round, unbudded, the same phenotype as overexpression of *CDC24* (331).

The ELM server predicts that Cdc24-Tyr200 could be part of the YXX Φ signal that interacts with the adaptor protein (AP) complex during endocytosis. GFP-Cdc24-Y200Fp exhibits wild-type targeting to polarized growth sites; however, as discussed in **Chapter 3** for GFP-Cdc24-S697Ap and in *tos2 Δ skg6 Δ* mutants, it is localized at the mother-bud neck of pre-anaphase cells abnormally. This may suggest that endocytic recycling of Cdc24p to the bud tip plays a role in polarized maintenance in conjunction with anchoring at the plasma membrane through an interaction with Tos2p. The CH domain of Vav also interacts with LyGDI (102); therefore, integration of phosphorylation and protein-protein interactions (see **Table 9**) in the CH domain could be a mechanism through which the activity and localization of Cdc24p, and therefore of Cdc42p, is regulated and should be investigated further, as little is known about the function of the CH domain.

The amino-terminus of the Rho1p GEF, Tus1p, is phosphorylated in G₁ by Cdc28p-Cln2p (156). This event leads to the activation of Rho1p at incipient bud sites. Hyperphosphorylation of Cdc24p is also correlated with high levels of Cln2p in G₁ and occurs *in vitro* with Cdc28p-Cln2p, but not Cdc28p-Clb2p (190). Rga2p, Boi1p and Boi2p, but not Bem1p, are also phosphorylated *in vitro* with Cdc28p-Cln2p (190). Inhibition of Cdc28p activity in G₁, but not actin depolymerization, prevented the targeting of Bem1p, Boi1p, Boi2p and Rga2p to

the incipient bud site (190). Inhibition of Cdc28p activity after polarized proteins were targeted also results in the loss of polarization of Boi2p, Bem1p and Cdc42p (190). Taken together, this suggests that phosphorylation of Rho GEFs in G₁ by Cdc28p-Cln2p could be a common mechanism to target them to incipient bud sites to activate Rho GTPases. Additionally, phosphorylation of GAPs and other polarity proteins, in a cell cycle-dependent manner, is likely to be the primary means by which these proteins are targeted and maintained at sites of polarized growth.

Lack of phosphorylation of Cdc24p at Ser697 in G₁ by Cdc28p-Cln2p, leading to a loss of maintenance at sites of polarized growth, is a probable explanation for the double band of GFP-Cdc24-S697Ap at the mother-bud neck pre-anaphase (see **Chapter 3**). This likely represents protein that was not maintained at the tip of the emerging bud (mother cell ring) and protein that was not maintained at the tip of the growing bud (daughter cell ring). It is probable that the septin collar, acting as the diffusion barrier (see **Diffusion Restriction**) between the mother and daughter cells, is located within these rings, which could be tested by utilizing CFP- Cdc24-S697Ap and YFP-Cdc12p.

The fact that GFP-Cdc24-S697Ap complements a *cdc24-4^{ts}* (see **Chapter 3**) is consistent with our results that ~30% of small-budded cells exhibit this aberrant localization. This suggests that phosphorylation of the remaining five CDK consensus phosphorylation sites can frequently achieve targeting and maintenance of GFP-Cdc24-S697Ap. The remaining five CDK sites in Cdc24p

should be examined, alone and in combination, to determine their contribution to targeting and maintenance of Cdc24p at sites of polarized growth.

Cdc24p is also phosphorylated by the PAK, Cla4p (34, 106). Cdc24p has two potential PAK sites, Ser198 within the CH domain, and Ser729, within the targeting and anchoring domain. Phosphorylation of these residues by Cla4p is proposed to lead to removal of Cdc24p from sites of polarized growth by decreasing its affinity for Bem1p (34). Analysis of the effects on Cdc24p localization by mutation of these sites could provide valuable information about maintenance of Cdc24p at sites of polarized growth.

Consensus phosphorylation sites were identified by ELM analysis (see **Table 9**) that could be recognized by Tor1-2p, Yck1-2p, Ygk3p Tpk1p and Cdc5p. These kinases have essential roles in many signaling pathways that Cdc42p, and therefore Cdc24p, are associated with. Tor1p and Tor2p, PI kinases, and Tpk1p, a cAMP-dependent kinase, are involved in nutrient responses [reviewed in (316)] and Tor2p in actin cytoskeleton polarization (180). Yck1p and Yck2p are plasma membrane-associated kinases required for morphogenesis (254), and Yck1p initiates translation of the *ASH1* bud-targeted mRNA (227). Yck2p has a role in maintenance of cell polarity (245), and with Yck1p, a role in morphogenesis (254). Ygk3p, a member of the GSK-3 family, has recently been shown to play a role in phosphorylation-mediated degradation of Rho4p (292) and might play a role in degradation of Cdc24p, if this kinase does phosphorylate Cdc24p. Cdc5p, a Polo-like kinase, could additionally

integrate signals related to cytokinesis (126). Little is currently known about the role cell-cycle dependent phosphorylation has on the localization and activity of Cdc24p; however, research presented in, and discussed within, this dissertation indicates that this is important to our understanding of how GEFs regulate GTPases.

Regulation of the Cdc42p Polarity Regulator:

The RhoGEF Cdc24p as a Scaffold

The ability of Cdc42p to cycle between GTP- and GDP-bound states is essential for its function (17, 95, 135). The GTP-hydrolysis requirement is proposed to be essential because it permits the continuous interaction of GDP-bound Cdc42p with Cdc24p, which could be important for effector protein localization and/or targeting (135). The only function currently ascribed to Cdc24p is GDP-GTP exchange for Cdc42p; however, the catalytic DH domain only comprises approximately 20% of the protein. The remaining 80% of the protein contains multiple ligand motifs for interaction with a wide range of proteins (see **Table 7**) and could participate in effector targeting to Cdc42p.

Multicopy suppressors of *cdc24-11* (cannot be suppressed by *CDC42* alone) were sought in a screen to address other potential functions of Cdc24p (23). *MSB1*, *MSB3*, *MSB4* (predicted transmembrane proteins with unknown function) and the amino-terminal 369 amino acids of the PAK *CLA4* were identified, in conjunction with *CDC42*, as suppressors of *cdc24-11*.

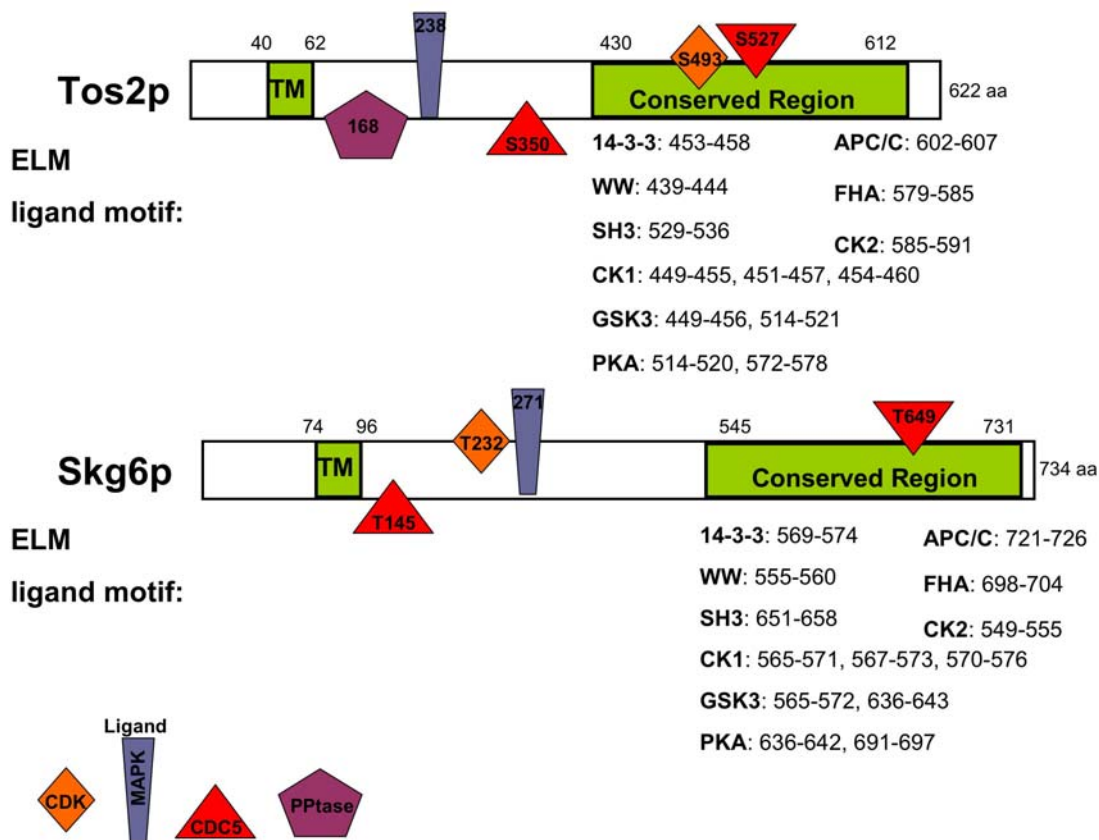
Overexpression of *CDC42* with the *CLA4* allele or *MSB1* in a strain expressing low levels of Cdc24p resulted in polarization of the cytoskeleton. These cells were able to form multiple, elongated buds, but were unable to form colonies, suggesting that Cdc24p could have an essential function in addition to its GEF function during bud emergence. What this function might be is still unknown. Because it is likely that the amino-terminus of Cdc24p has functional, in addition to regulatory, roles, a yeast two-hybrid screen utilizing the amino-terminus may provide insight into this function by identifying proteins that interact with it.

Regulation of the Cdc42p Polarity Regulator:

Maintenance of the Cdc24p GEF at Polarized Growth Sites

SKG6 was originally identified as a dosage suppressor of the cell wall defects of a *kex2Δ gas1Δ* mutant (297) and of *cdc42-118* (75). *SKG6* shares 35% sequence homology with *TOS2*, most of which is within their carboxyl-termini (see **Figure 20**). *TOS2* is unable to suppress the cell wall defects of a *kex2Δ gas1Δ* mutant, suggesting that they are not functionally redundant. Little is known about the function of either protein. An ELM analysis was performed on both proteins. Ligand domains that were conserved are listed under the conserved region and ligand domains that were not conserved between the proteins are highlighted based on function (CDK, MAPK, Cdc5p, and phosphatase). Mutations of these regions and determination of their affect on

Figure 20: Ekaryotic Linear Motif (ELM) predictions for Tos2p and Skg6p.



Cdc24p localization will be important for understanding how they function to anchor Cdc24p at sites of polarized growth.

SKG6 and *TOS2* have also been identified as dosage suppressors of a *gic1 gic2-Ts* mutant and overexpression of either Skg6p or Tos2p resulted in cells with septum, cytokinesis and septin localization defects (86). In addition to the evidence presented in **Chapter 3** and Toenjes, et al. (294), this could suggest that Cdc24p maintenance at the mother-bud neck is also essential for activation of Cdc42p during cytokinesis. Skg6p has an additional role during cytokinesis, as an inhibitor of Hof1p function (86), which could explain why overexpression of Skg6p had a greater effect on septum, cytokinesis and septin localization defects than overexpression of Tos2p. It has also been proposed that Tos2p could function as a regulator of Rho1p and Cdc42p signaling because Tos2p interacts with Pkc1p, an effector protein of Rho1p (75).

Cdc24p also has two proposed Group IV WW domain ligand sites from the ELM analysis [Ser697 and Thr704, (see **Table 7**)]. WW domains are 40 aa domains that contain two conserved Trp (W) residues that interact with proline-rich regions of their targets (286). Several consensus sequences have been identified for WW ligands: Group 1 – PPXY, Group 2 – PPLP, Group 3 – PGM, and Group 4 – [ST]P, which is phosphorylation dependent [reviewed in (259), ELM]. 2-D gel analysis of GFP-Cdc24-647-854-S697A, T704Ap is currently underway to determine if both sites are subjected to phosphorylation.

SGD annotates six proteins that have a WW domain. Prp40p and Urn1p are RNA splicing factors, Set2p is a histone methyltransferase and the function of Wwm1p is unknown. Rsp5p is an E3 ubiquitin ligase that recognizes the motif (L/P)PXY (259) and Ess1p is a peptidyl-prolyl *cis/trans* isomerase (PPlase) that recognizes phosphorylated Ser or Thr residues immediately followed by Pro (319). Ess1p and Rsp5p compete for binding to the C-terminal domain (CTD) of RNA pol II, potentially regulating the activity (Ess1p) and amount of RNA pol II (Rsp5p) (318).

Cdc24p has not been identified as a substrate of Rsp5p and does not have the (L/P)PXY motif; however, a screen to identify Rsp5p substrates found that 28% of high-confidence targets did not have the consensus sequences typically associated with Rsp5p (110). Preliminary results indicate that Cdc24p is conjugated to ubiquitin (K. Cole and D. Johnson, unpublished results); therefore, Cdc24p should be assessed for the addition of ubiquitin in an *rsp5Δ* mutant. Two point mutations in Ess1p, W15R in the WW domain and G127D in the PPlase domain, result in a *ts* phenotype (319) and could be used to determine if Cdc24p interacts with the WW domain of Ess1p and if PPlase function is essential for its localization and interaction with other proteins.

The research presented in this dissertation provides insight into how Cdc42p is regulated during the cell cycle through complex formation with its negative regulator, Rdi1p, and how it is activated only at sites of polarized growth by the targeting and maintenance of its activator, the GEF Cdc24p. It is important that future research connect cell cycle-dependent phosphorylation events to the activity and localization of Cdc42p's regulators so that we can fully understand the central regulator of cell polarity.

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